Lipid Transport by ABC Proteins
Studies on MDR1 P-Glycoprotein and Breast Cancer Resistance Protein

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Summary

In eukaryotic cells, the lipid species are frequently distributed asymmetrically between the plasma membrane leaflets. Phosphatidylserine (PS), in particular, often exhibits a distinct transverse asymmetry, being restricted almost exclusively to the inner leaflet.
In the past years, several proteins were suggested to transport lipids between the leaflets of a membrane, and to potentially influence transverse lipid asymmetry and related cell properties.
This thesis focuses on outward transport of fluorescent (C6-NBD-) lipid analogs and endogenous lipids by the Multidrug Resistance 1 P-Glycoprotein (MDR1 Pgp), a member of the ATP binding cassette (ABC) transporter superfamily. Interestingly, MDR1 Pgp has been suggested to exhibit an unusually broad substrate specificity. Here, the anionic PS was of particular concern, although previously reported not to be an MDR1 Pgp substrate.
In a human gastric carcinoma cell line (EPG85-257) overexpressing *MDR1*, outward transport of phosphatidylcholine, phosphatidylethanolamine, glucosylceramide and sphingomyelin analogs via MDR1 Pgp was confirmed using fluorescence spectroscopy. In addition, decreased accumulation of analogs of diacylglycerol and ceramide suggest MDR1 Pgp mediated transport of these lipid species.
Upon intracellular labelling with C6-NBD-PS using a novel approach, significantly increased outward transport of this analog in *MDR1* overexpressing cells could be attributed to MDR1 Pgp by employing specific inhibitors.
In a flow cytometry setup, the exposure of endogenous PS on the outer plasma membrane leaflet was significantly elevated in *MDR1* overexpressing cells compared to controls. Reduction of PS exposure by an MDR1 Pgp inhibitor suggests transport of endogenous PS by MDR1 Pgp.
Transport of C6-NBD-PS was furthermore characterized here in four additional cell lines of different species and tissue origin with varying synthesis levels of MDR1 Pgp.
Besides MDR1 Pgp, the ABC half-size transporter Breast Cancer Resistance Protein (BCRP) is possibly also involved in transport of C6-NBD-PS and in increased exposure of endogenous PS, as found in a *BCRP* overexpressing EPG85-257 subline.

Keywords

ABCB1

ABCG2

Annexin

Flippase

KPG7

Lipid Asymmetry

LLC-PK1

MDCK II

MF

Plasma Membrane

Zusammenfassung

In eukaryotischen Zellen sind die Lipidspezies häufig asymmetrisch zwischen den Hälften der Plasmamembran verteilt. Insbesondere Phosphatidylserin (PS) weist oft eine ausgeprägte transversale Asymmetrie auf, da es fast ausschliesslich auf die innere Hälfte der Plasmamembran beschränkt ist.
In den letzten Jahren wurden mehrere Proteine diskutiert, die Lipide zwischen den Membranhälften transportieren und möglicherweise die transversale Lipidasymmetrie sowie damit verbundene Zelleigenschaften beeinflussen.
Im Mittelpunkt der vorliegenden Promotion steht der Auswärtstransport fluoreszierender (C6-NBD-) Lipid-Analoga und endogener Lipide durch das Multidrug Resistance 1 P-Glycoprotein (MDR1 Pgp), das der ATP Binding Cassette (ABC) Transporter Superfamilie angehört. Interessanter Weise wird für MDR1 Pgp eine ungewöhnlich breite Substratspezifität angenommen. Das anionische Lipid PS war hier von besonderem Interesse, obgleich es in vorhergehenden Arbeiten nicht als MDR1 Pgp Substrat betrachtet wurde.
Der Auswärtstransport von Phosphatidylcholin-, Phosphatidylethanolamin-, Glucosylceramid- und Sphingomyelin-Analoga durch MDR1 Pgp konnte in einer humanen Magenkarzinomlinie (EPG85-257), die *MDR1* überexprimiert, mittels Fluoreszenzspektroskopie bestätigt werden. Zudem legt die verringerte Akkumulation von Diacylglycerol- und Ceramid-Analoga den Transport dieser Lipidspezies durch MDR1 Pgp nahe.
Im Anschluß an die intrazelluläre Markierung mit C6-NBD-PS mittels eines neuen Verfahrens konnte der signifikant erhöhte Auswärtstransport dieses Analogons in *MDR1* überexprimierenden Zellen durch Verwendung spezifischer Inhibitoren MDR1 Pgp zugeschrieben werden.
In flusscytometrischen Versuchen war die Exponierung von endogenem PS auf der äusseren Membranhälfte von *MDR1* überexprimierenden Zellen signifikant höher als in Kontrollzellen. Verringerung der PS-Exponierung durch einen Inhibitor von MDR1 Pgp deutet auf den Transport von endogenem PS durch MDR1 Pgp hin.

Zusätzlich wurde hier der Transport von C6-NBD-PS in vier weiteren Zellinien mit verschiedener Spezies- und Gewebezugehörigkeit charakterisiert, die unterschiedliche Mengen an MDR1 Pgp synthetisieren.
Wie Experimente in einer *BCRP* überexprimierenden EPG85-257-Sublinie nahelegen, ist ausser MDR1 Pgp möglicherweise ebenfalls der ABC Halb-Transporter Breast Cancer Resistance Protein (BCRP) am Transport von C6-NBD-PS und an der verstärkten Exponierung von endogenem PS beteiligt.

Schlagworte

ABCB1
ABCG2
Annexin
Flippase
KPG7
Lipidasymmetrie
LLC-PK1
MDCK II
MF
Plasmamembran

1 Introduction

In lipid membranes, the transverse passage from one leaflet to the other is slow for most lipid species. However, while crossing of the hydrophobic membrane core is energetically unfavorable for the hydrophilic headgroup of a lipid by itself, this can be extensively facilitated through lipid transporting proteins.

Since changes in the asymmetrical distribution of a lipid species between the membrane leaflets can influence membrane curvature and fusion competence, protein association and activity, as well as various biochemical pathways, these transport proteins may possess regulatory key functions, e.g. in processes like apoptosis, multidrug resistance or phagocytosis. In particular, transport of the aminophospholipid phosphatidylserine and ensuing alterations in its asymmetrical distribution could be of fundamental consequences for the cell.

However, although some lipid transport proteins have been identified in the last years, the protein vehicles responsible for many lipid transport phenomena have not been distinguished yet. The ATP binding cassette (ABC) protein superfamily comprises transporters for a whole bandwidth of organic and inorganic compounds. Several ABC proteins are specific for particular amphiphilic substrates, while more unspecific substrate transport is found in a small number of ABC proteins only.

The full-size ABC transporter Multidrug Resistance 1 P-glycoprotein (MDR1 Pgp), discovered in 1976, has for a long time been considered a rather unspecific transporter for cationic or electrically neutral amphiphiles, among them cytostatic drugs and several lipid species.

In contrast to MDR1 Pgp, little is known about the recently discovered half-size ABC transporter Breast Cancer Resistance Protein (BCRP) and its substrate preferences.

In the present work, outward transport of lipid analogs and endogenous lipids by the ABC proteins MDR1 Pgp and BCRP is assessed with emphasis on the anionic lipid phosphatidylserine.

In chapter 2, some general features of membranes are regarded, with a focus on lipid transverse movement. Chapter 3 provides an overview of the ABC superfamily and its involvement in lipid transport, and chapter 4 sums up the aim of the present study. Following the materials and methods section in chapter 5, the results are presented in chapters 6 to 8, in which the data have been grouped according to the transport proteins on the one hand, and the utilized cell lines on the other hand. Chapters 6 to 8 each contain a brief discussion. Conclusions of the main results are discussed in the context of the whole work in chapter 9 before an outlook is attempted in chapter 10.

2 Biological Membranes

2.1 Structure and Function

Biological membranes are at a time physico-chemical barriers, and places of material and informational exchange between reaction compartments within a cell and between the cell and its environment. This is achieved by the unique architecture of membranes (Singer, 1972), formed by lipids, proteins, and a low percentage of either lipid- or protein-associated carbohydrates (Fig.1): Membrane lipids are organized in a bilayer with the hydrophilic headgroups of glycerophospholipids and sphingolipids oriented towards the aqueous media on either side of the membrane, and the hydrophobic fatty acid chains oriented towards the interior of the membrane. Membrane proteins either span the lipid bilayer (integral membrane proteins) or are loosely associated with it (peripheral membrane proteins).

While the proteins fulfill a number of specialized functions in the membrane, e.g. as enzymes, transporters, structural elements or receptors, lipids supply a strong yet flexible matrix for the membrane, however, their role goes far beyond this structural function: Particular lipid species and mixtures are involved in the regulation of protein activity, signalling, enzymatic reactions, cell-cell interactions, membrane sorting and vesicular transport (Gennis, 1989), (Zachowski, 1993).

2.2 Lipids

Glycerophospholipids, sphingolipids, and sterols comprise the majority of the lipids found in the plasma membrane, surrounding the eukaryotic cell (Gennis, 1989).

Glycerophospholipids are made up of a glycerol backbone esterified with fatty acids at C1 and C2, and a headgroup at the phosphoryl group (Fig.2).

Length and degree of saturation of the fatty acid chains, as well as size, charge and chemical nature of the headgroup determine the properties of a glycerophospholipid. The relative size of fatty acid chains towards the headgroup determines the molecular shape of the lipid, which can influence the curvature of a membrane (Cullis, 1979) (Fig.3).

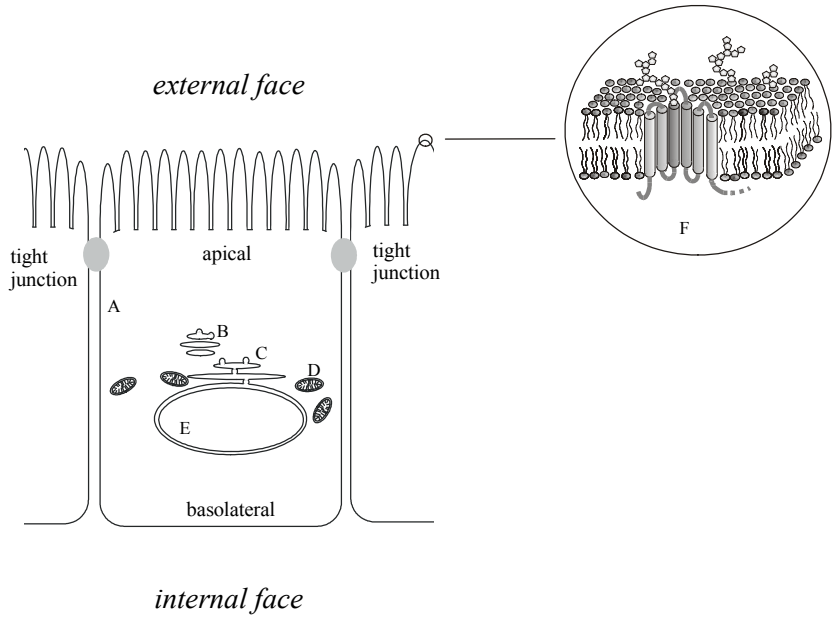

Fig.1: Membranes within an eukaryotic cell.

While the cell is surrounded by a single membrane (plasma membrane, A), organelles can have one (Golgi apparatus, B; endoplasmic reticulum, C) or two membranes (mitochondria, D; nucleus, E) separating them from the cytosol. In an epithelial cell, tight junctions laterally separate the apical from the basolateral macrodomain of the outer plasma membrane leaflet, while the inner leaflet is continuous (van Meer, 1986). In the case of a polarized epithelial cell in the intestine, this structural feature allows uptake of nutrients over the highly folded apical membrane facing the organ lumen, and nutrient export to the internal face, which is directed towards the blood stream. F shows a schematic membrane section:
The transmembrane helices (grey cylinders) of an integral protein reach through the lipid bilayer, carbohydrates (light grey hexagons) are attached to either lipid or protein moieties on the extracellular leaflet.

Although similar to glycerophospholipids in many physicochemical properties, sphingolipids do not originate from glycerol, but are N-acyl fatty acid derivatives of long-chain amino alcohols, in which the C1 hydroxyl group can be replaced by a phosphocholine or phosphoethanolamine (sphingophospholipids), or in glycosphingolipids by a single sugar residue (cerebrosides) or a sialic-acid-complexed sugar residue (gangliosides) (Fig.2). Sterols are tetracyclic molecules derived from cyclopentanophenanthrene, varying in the side chains and the localization of double bonds (Fig.2).

The glycerophospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), the sphingolipid sphingomyelin (SM) and the sterol cholesterol are the major lipids found in eukaryotic membranes (Gennis, 1989) (
Table 1).

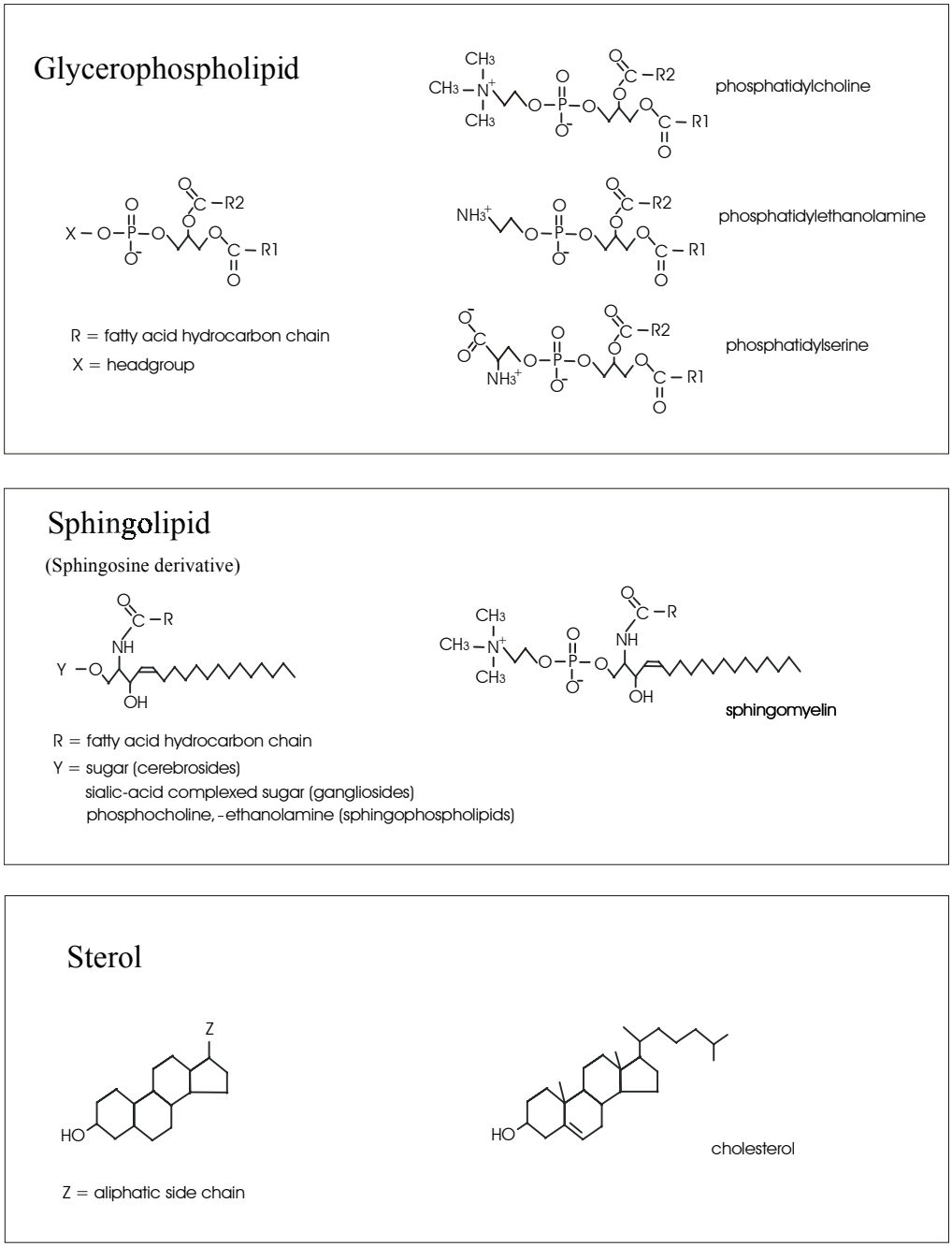

Fig.2: Major lipid classes in the plasma membrane of eukaryotic cells.

The common feature of the heterogeneous group of lipids is their good solubility in organic solvents, and a limited solubility in water. Apart from the lipids occurring in membranes, triacylglycerols (serving as energy stores) and steroids (including many vitamins and hormones) are lipids of biological importance.

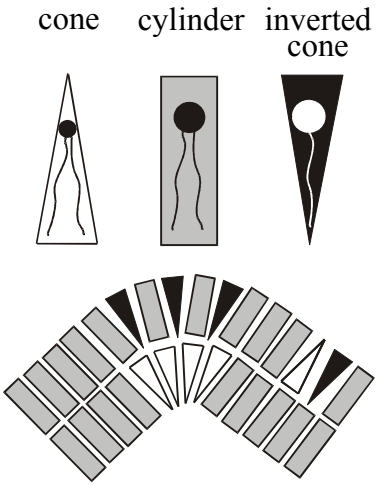

Fig.3: Lipid molecular shapes and membrane curvature.

In cone-shaped lipids, the fatty acid chains dominate in size over the headgroup (PE, PA, DG, Cer). The headgroup is similar in size to the fatty acid chains in cylindrical lipids (PC, PS), while it dominates over the fatty acid chains in inverted-cone-shaped lipids (lysolipids,(SM)). Below, the influence of lipid shapes on the curvature of the membrane is shown schematically (adapted from (Sprong, 2001)).

Table 1: Phospholipid composition of plasma membranes and intracellular membranes in rat and human cells.

	Fraction of total phospholipids (in%)				
	human erythrocyte plasma membrane*	rat hepatocyte plasma membrane	rat hepatocyte mitochondrial membrane	rat hepatocyte nuclear membrane	rat hepatocyte Golgi membrane
PE	28	23	35	13	20
PC	29	39	40	55	50
PI	1	8	5	10	12
PS	15	9	1	3	6
SM	26	16	1	3	8
Cardiolipin	-	1	18	4	1

Data from (Daum, 1985), * (Zwaal, 1975).

2.3 The Complex Composition of Membranes

It is the particular composition that characterizes specialized membranes. The protein-to-lipid ratio can range between 20 and 80% (Gennis, 1989), and the relative abundance of the protein and lipid species is variable, depending on the respective physiological function of the membrane: The plasma membrane of Schwann cells (myelin sheath), electrically insulating neuronal axons, has for instance a protein content of only about 20% and is rich in the otherwise rare sphingolipid class of gangliosides (Devaux, 1985). A differently specialized membrane, the mitochondrial inner membrane, which is central to the cell respiration process, contains about 75% protein (mostly enzymes of the respiratory chain), little cholesterol, but a high percentage of cardiolipin, a dimeric glycerophospholipid typical for prokaryotes (Daum, 1985).

Asymmetrical lipid distribution

However, differences in composition can even be found in the lateral macrodomains of one and the same membrane: In polarized epithelial cells, e.g. in the intestinal epithelium (Fig.1), tight junctions serve as diffusion barriers (Dragsten, 1981) between the apical exoplasmic domain rich in glycosphingolipids, and the basolateral exoplasmic domain containing an increased amount of phosphatidylcholine (Simons, 1988). Lateral membrane macrodomains are also found in sperm cells and retinal rods (Gennis, 1989).
In the past years, evidence for lateral microdomains (so-called rafts) in membranes has been presented. According to the raft concept, dynamic clusters of sphingolipids and cholesterol form in the membrane, and selectively support or prevent association of specific proteins (Simons, 1997). Rafts have been suggested to be involved in membrane sorting, in the formation, movement, docking and fusion of vesicles, in signalling and in endocytic uptake (Ikonen, 2001).
In many eukaryotic cells, differences in lipid composition occur also between the inner and outer leaflet of their membranes (transverse asymmetry) (Zachowski, 1993). In the plasma membranes of human erythrocytes, PC and sphingolipids dominate in the outer (exoplasmic) leaflet, PE and PS in the inner (cytoplasmic) leaflet (Fig.4), similar to the less abundant lipids PI, phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-4,5-bisphosphate (PIP2), and phosphatidic acid (PA) (Zachowski, 1993).

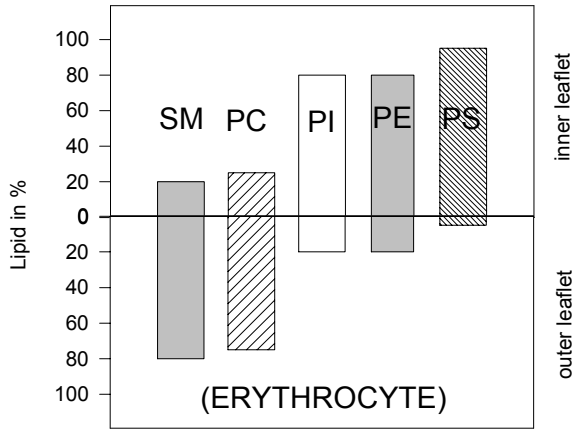

Fig.4: Transverse distribution of endogenous lipid species in the plasma membrane of the human erythrocyte.

Sphingomyelin (SM) and phosphatidylcholine (PC), carrying a phosphocholine headgroup, are mostly found in the outer plasma membrane leaflet, while the major portion of phosphatidylinositol (PI) and the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) resides in the inner plasma membrane leaflet (Zachowski, 1993). The human erythrocyte is frequently employed to determine the transverse distribution of endogenous lipids, since it disposes of a plasma membrane, but not of internal membranes.

For cholesterol, an asymmetric distribution over the leaflets of the plasma membrane is not conclusively established today (Pomorski, 2001).

In various eukaryotic cells originating from different species and tissues, the transverse lipid distribution is generally similar to the pattern observed in the human erythrocyte (Zachowski, 1993): In virtually all cells studied, SM is restricted to the outer, and PS to the inner leaflet of the plasma membrane. PC often dominates in the outer, and PE in the inner plasma membrane leaflet, but these two lipids appear to be less strictly limited to one particular localization than SM and PS.

In several physiologically altered cells, however, the originally highly asymmetric distribution of PS undergoes major changes. For example, increased amounts of PS appear on the outer leaflet of the plasma membrane of stimulated thrombocytes (Bevers, 1982) and of some tumorigenic cell lines (Utsugi, 1991).

In apoptotic cells, the asymmetry of PS is largely lost (Fadok, 1992).

An asymmetric transverse distribution has been reported for several lipid species in the membranes of a number of organelles, e.g. mitochondria and sarcoplasmic reticulum (Zachowski, 1993). However, as the isolation of intracellular organelles involves the risk of membrane perturbation, there is not an abundant amount of firmly established data available concerning organelle lipid asymmetry.
In the ER, the organelle mainly responsable for lipid synthesis, the enzymes involved in lipid formation are asymmetrically located in the two membrane leaflets (Sprong, 2001), which necessitates lipid transport across the membrane. Due to ATP-independent, bidirectional protein-mediated transport, the distribution of lipids is assumed to be near to symmetric in this organelle (Bishop, 1985), (Herrmann, 1990), (Buton, 1996).

Possible functions of lipid transverse asymmetry

The asymmetric distribution of lipids across the membrane leaflets appears to be an active property of eukaryotic cells, generated by the interplay of transversely asymmetric lipid synthesis and breakdown, and of selective transport (Sprong, 2001), balancing passive transbilayer movement. Depending on their physicochemical properties, different lipid species can promote membrane association and fusion (e.g. lipids with negatively charged headgroups exhibiting a small hydration radius (Devaux, 1991)), form domains which are preferred by a particular subset of proteins (e.g. lipids with fatty acid chains matching protein transmembrane domains in length (Mouritsen, 1984) or allowing a certain degree of motional freedom), and permit or inhibit membrane bending according to their molecular shape (Cullis, 1979). Moreover, they are able to influence the activity of specific membrane proteins (e.g. by interaction with regulatory domains (Sandermann, 1978)), or participate in numerous biochemical pathways (e.g. enzymatic or signalling routes (Spiegel, 1996)).
In comparison to PC and SM, the headgroups of PS and PE exhibit a comparatively small hydration radius, facilitating negative curvature and the approximation of membranes (2.2).

In addition, due to the negative charge of PS, membranes rich in this lipid can form non-bilayer structures in the presence of Ca^{2+}, excluding water (Portis, 1979) and facilitating fusion. Therefore, the presence of PS on the outer leaflet of the plasma membrane does not seem to be advantageous for most cells, and has been shown to cause cell adherence (Schlegel, 1985). Since PS might facilitate endo- and exocytosis of membrane vesicles (Devaux, 1991), it would be beneficial for PS to be localized in the inner leaflet of the plasma membrane and the cytoplasmic leaflet of intracellular vesicles. Macrophage recognition of physiologically altered cells by detecting PS exposure (Marguet, et al., 1999) via a PS specific receptor (Fadok, 2000) has probably evolved consequently to the setting-up of transverse lipid asymmetry as a characteristic of eukaryotic plasma membranes. For the coagulation cascade, on the other hand, clotting of platelets is desired, and PS becomes exposed to the outer leaflet upon platelet activation (Bevers, 1999).
The strong preference of SM for the outer leaflet of the plasma membrane might be connected to the signalling function of Cer (chapter 9), for which SM serves as a metabolic precursor. Separation of the enzyme (SMase) from the bulk of the substrate could allow more precise regulation.
PC, one of the more abundant lipids in mammalian plasma membranes, is probably a structural membrane compound in the first place. It appears to be little involved in processes like signalling or fusion, possibly reflected by its moderate asymmetry.
It is equivocal what exactly compels cells to establish membranes with two differently composed leaflets. Yet, combined with the presence of regulatory mechanisms such as lipid flippases, this could provide the cell with a tool to modulate curvature and fusion competence of membranes, as well as association and activity of membrane proteins.

2.4 Lipid Movement and Transport

Lateral translation of lipids is relatively fast (10^{-9} - 10^{-7} cm^2/s) (Henis, 1993).
In contrast, the transverse motion (flip-flop) of glycerophospholipids and glycosphingolipids across a pure lipid bilayer is extremely slow ($t_{1/2}$ > 11 d for dioleoyl-PC in pure dioleoyl-PC membranes) (Rothman, 1975), since passage of the hydrophilic lipid headgroup across the hydrophobic membrane core is energetically unfavorable.

Lipids with a small, uncharged headgroup as diacylglycerol (DG) or ceramide (Cer), respectively with a headgroup in the non-ionized form and a small hydration shell as phosphatidic acid (PA), have a higher flip-flop rate than lipids carrying a large and/or charged hydrophilic headgroup (Zachowski, 1993) such as PS. In the case of a lipid with a headgroup which can be neutralized by protonation, the distribution to the inner or outer membrane leaflet may thus depend on the pH on either side of the membrane (Hope, 1989), however, this is probably of little biological significance (Zachowski, 1993).
With the hydrophobicity of its fatty acid chains, the flip-flop rate of a lipid increases (Sprong, 2001).
Since certain fatty acid chains are found more abundantly in association with particular headgroups (e.g. in human erythrocytes longer, more unsaturated chains have been observed for PS than for PC) (Myher, 1989), an asymmetric distribution of the headgroups over the leaflets of the membrane might be related to an asymmetric transverse distribution of the fatty acid chains.
The general presence of proteins in the membrane can increase the velocity of transverse motion ($t_{1/2}$ = 1 h for dioleoyl-PC in dioleoyl-PC+glycophorin membranes) (de Kruijff, 1978), but only when lipid transporting proteins are present, a maximum is reached ($t_{1/2}$ = 5 minutes for endogenous PC in rat liver ER) (van den Besselaar, 1978), (Bishop, 1985).

2.5 Lipid Transporters

In eukaryotes, protein mediated transport of lipids has been found in the plasma membranes and in the membranes of some organelles. Membrane proteins which do not consume ATP can facilitate flip-flop of a lipid in the direction of its electrochemical and concentration gradient, e.g. by supplying a pathway for the hydrophilic headgroup through the hydrophobic core of the membrane (Raggers, 2000).
When a lipid is actively transported, involving the hydrolysis of ATP, this additionally allows movement against the electrochemical or concentration gradient of the lipid.

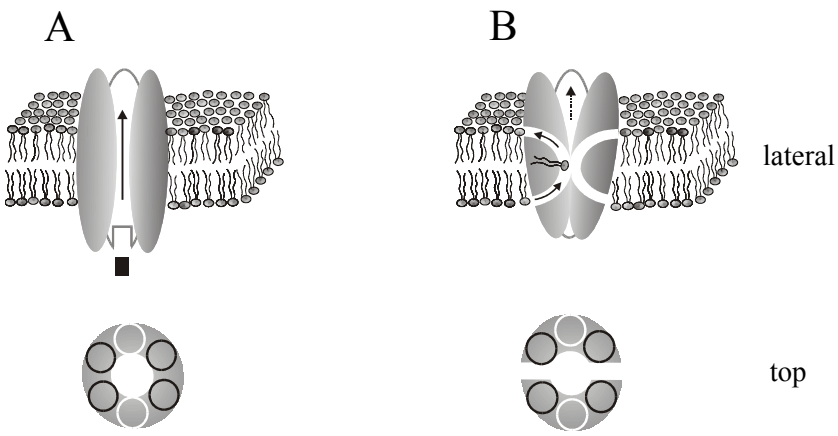

Fig.5: Outward transport of a substrate across a membrane.

A: Hydrophilic pore model. From the aqueous phase, a substrate (black rectangle) interacts with the recognition site of a transport protein and reaches the aqueous medium on the other side of the membrane via a hydrophilic channel. B: Flippase/vacuum cleaner model. A substrate partitions into the inner leaflet of the membrane and gains access to the transport protein by lateral openings between the transmembrane domains (see top view). It is then transported to the outer membrane leaflet (flippase, black arrow) or into the outer aqueous medium (vacuum cleaner, dotted arrow). For simplicity, 6 instead of 12 putative transmembrane helices are shown here (top view).

A classical transporter forming a hydrophilic pore according to Higgins and Gottesman (Higgins and Gottesman, 1992) possesses an enzyme-like substrate recognition site determining the respective substrate specificity (Fig.5). The substrate interacts with the transport protein from the aqueous phase, and is shielded from the hydrophobic environment during its passage through a hydrophilic pathway provided by the transport protein.

In contrast to this mechanism, Higgins and Gottesman suggested transport of amphiphilic substrates to occur via a flippase/vacuum cleaner mechanism (Fig.5). There, the substrate must partition into the membrane before interacting with the transport protein. The interaction of the substrate with the substrate binding site can be of secondary importance. The substrate is flipped across the membrane and is either released into the membrane (flippase) or into the aqueous medium (vacuum cleaner). The different steps of the transport process are triggered by conformational changes of the protein upon ATP binding, hydrolysis or release.

Although the map of proteins responsible for lipid transport is filling in, many events of protein mediated transport of lipids can still not be assigned unambiguously to a particular protein. Below, a number of important transmembrane transport processes are described.

2.5.1 Aminophospholipid transport in the plasma membrane

In the plasma membrane of virtually all eukaryotes, the aminophospholipids PS and PE are selectively and rapidly ($t_{1/2}$ approx. 5 minutes) transported from the outer to the inner leaflet of the membrane in an ATP-dependent process (Zachowski, 1993). As Heinrich et al. have shown, passive transmembrane transport of all lipid species and active inward transport of PS and PE are sufficient to establish the asymmetric lipid distribution found in the erythrocyte membrane (Heinrich, 1997). Although some candidate aminophospholipid translocases have been proposed (Schroit, 1987), (Auland, 1994), (Tang, 1996), so far reconstitution experiments have not yielded a protein exhibiting a similarly efficient aminophospholipid transport as seen in vivo.

2.5.2 Phospholipid transport in the ER

PC, PE and PS, synthesized on the cytoplasmic leaflet of the ER membrane (Cornell, 2000), (Kent, 1995), (Kuge, 1997), have to be transported to the lumenal membrane leaflet in order for the membrane to be equilibrated in the number of lipid molecules on either leaflet. ATP independent proteins appear to rapidly ($t_{1/2}$ = seconds to minutes) (Marx, 2000) transport glycerophospholipids (and with low affinity also sphingomyelin) bidirectionally across the ER membrane (Buton, 1996). Recently, transport active ER protein fractions have been isolated (Menon, 2000).

2.5.3 Glycosphingolipid transport in the Golgi

The glycosphingolipid Glucosylceramide (GlcCer), synthesized on the cytoplasmic leaflet of the Golgi, must be flipped to the lumenal leaflet in order to serve as a substrate for the synthesis of higher glycosphingolipids. The protein mediating this transport ($t_{1/2}$ approx. 5 minutes, J. Kubelt, unpublished results) , which is presumably ATP independent (Burger, 1996), has not been identified until now.

2.5.4 Phospholipid transport in the plasma membrane

Phospholipids are equilibrated across the plasma membrane of blood platelets and erythrocytes ($t_{1/2}$ approx. 5 minutes, (Nielsch, 2000)) when the intracellular Ca^{2+} concentration is increased (Comfurius, 1990).
A protein thought to mediate this bidirectional, ATP independent process (Basse, 1996), (Zhou, 1997) was termed *scramblase.* It is a member of the phospholipid scramblase protein family (Wiedmer, 2000). Besides erythrocytes and platelets, the scramblase is also active in a variety of other tissues. It is not resolved whether the exposure of PS during apoptosis is mediated by the scramblase as well (Bevers, 1999), another possible candidate being the ABC protein ABCA1 (Marguet, et al., 1999).

2.5.5 Lipid transport by ABC proteins

Recently, various lipid transport events could be directly or indirectly attributed to members of the ABC protein superfamily, which is described in detail in the next chapter.

3 The ABC Protein Superfamily

3.1 General Features

The ATP-binding cassette (ABC) protein superfamily comprises a large number of either import or export pumps (no bidirectional ABC transporters identified so far) in pro- and eukaryotes (Higgins, 1992). ABC protein functions range from the acquisition of nutrients (only in prokaryotes) and the excretion of waste products to regulation.
Generally, they are low capacity, but high affinity transporters, able to transport substrates against a concentration gradient up to more than 10 000 fold. Typically, ABC proteins are relatively specific for a particular set of substrates (MDR1 Pgp probably represents an exception in this respect). Substrates can be amino acids, sugars, inorganic ions, peptides, proteins, lipids and various organic and inorganic compounds. Structurally, ABC proteins have a typical organization of two cytoplasmic ATP binding cassettes (ABC domains), and two transmembrane (TM) domains consisting of six alpha-helices each. In some ABC proteins, deviating organizations of the domains can prevail (Fig.6) (Klein, 1999). The transmembrane domains vary considerably between different transporters, whereas the ATP binding domains are highly conserved (e.g. Walker motifs). The substrate specificity is believed to be determined by the transmembrane domains, including the loops connecting the individual helices (Higgins, 1992). Hydrolysis of ATP is required for substrate transport by all ABC proteins.

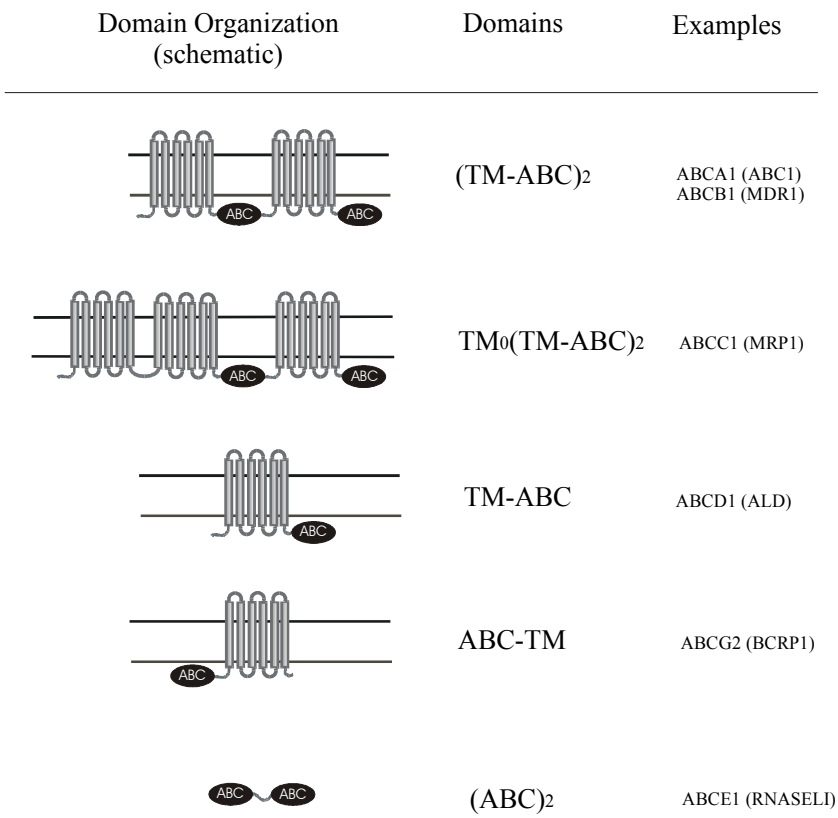

Fig.6: Domain organization in ABC proteins.

ABC domains (ABC) and transmembrane (TM) domains are the building blocks that ABC proteins are made up of. Depending on their domain arrangement, ABC proteins can be active as monomers, or have to form homo- or heterodimers in order to be functional (Klein, 1999).

The stochiometry of transport is estimated to be close to one substrate molecule for every hydrolyzed ATP molecule. Regulation of ABC proteins occurs mostly on the level of protein synthesis, but in some members of this subfamily, phosphorylation also plays a role (Higgins, 1992).

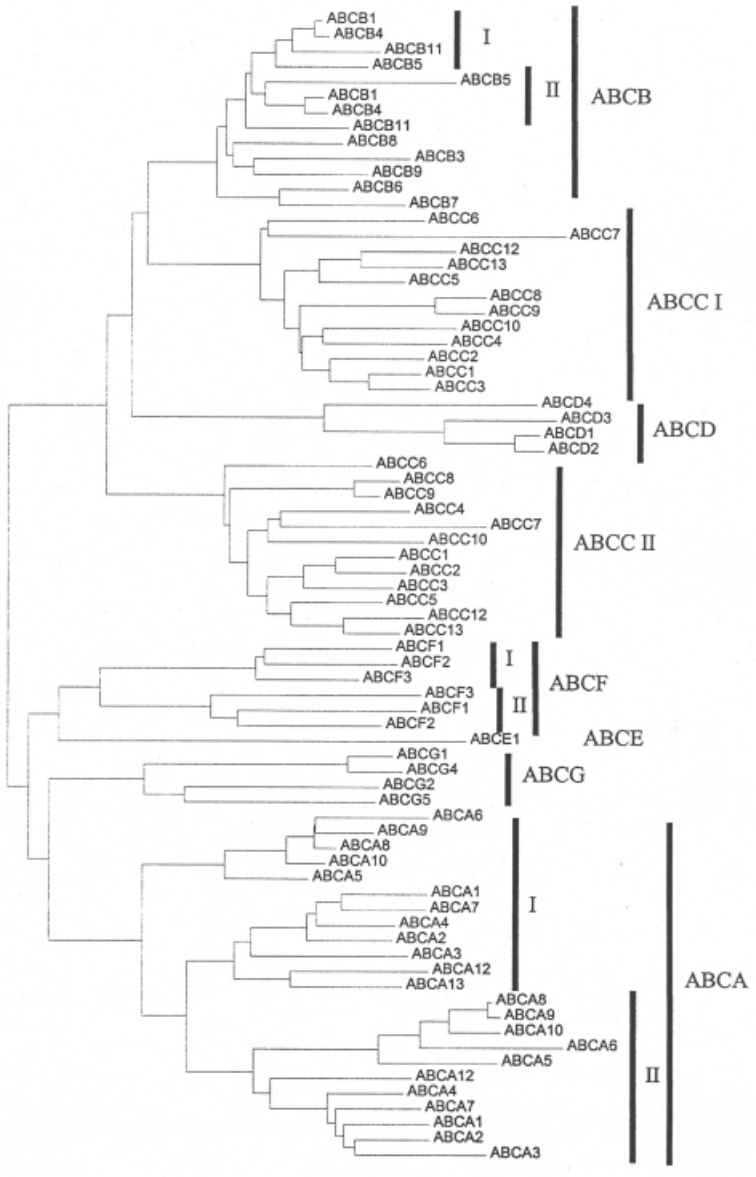

Figure 7: Genealogical tree of human ABC genes.

According to homology of the ABC domains (I: N-terminal, II: C-terminal), the human ABC genes are grouped here into the families (ABC)A to (ABC)G (taken from (Dean, 2001)).

Table 2: Human ABC proteins (names according to the guidelines for human gene nomenclature) (Müller, 2002).

Family (trivial name)	**ABCA (ABC1)**	**ABCB (MDR/TAP)**	**ABCC (CFTR/MRP)**	**ABCD (ALD)**	**ABCE (OABP)**	**ABCF (GCN20)**	**ABCG (WHITE)**
Protein (trivial name)	ABCA1 (ABC1)	ABCB1 (MDR1)	ABCC1 (MRP1)	ABCD1 (ALD)	ABCE1 (RNASELI)	ABCF1 (ABC50)	ABCG1 (WHITE)
	ABCA2 (ABC2)	ABCB2 (TAP1)	ABCC2 (MRP2)	ABCD2 (ALDL1)		ABCF2	ABCG2 (BCRP1)
	ABCA3 (ABC3)	ABCB3 (TAP2)	ABCC3 (MRP3)	ABCD3 (PXMP1)		ABCF3	ABCG3 ?
	ABCA4 (ABCR)	ABCB4 (MDR2/3)	ABCC4 (MRP4)	ABCD4 (PXMP1L)			ABCG4 (WHITE 2)
	ABCA5	ABCB5	ABCC5 (MRP5)				ABCG5 (WHITE 3)
	ABCA6	ABCB6	ABCC6 (MRP6)				ABCG8 (WHITE 4)
	ABCA7	ABCB7	ABCC7 (CFTR)				
	ABCA8	ABCB8 (M-ABC1)	ABCC8 (SUR1)				
	ABCA9	ABCB9	ABCC9 (SUR2)				
	ABCA10	ABCB10	ABCC10				
	ABCA12	ABCB11 (BSEP)	ABCC11				
	ABCA13		ABCC12				
members	12	11	12	4	1	3	5(+1?)

ABCR Retina Specific ABC Protein; ALD Adrenoleukodystrophy Protein; ALDL ALD-like; BCRP Breast Cancer Resistance Protein; CFTR Cystic Fibrosis Transmembrane Conductance Regulator; MDR Multidrug Resistance Protein; MRP Multidrug Resistance Protein; OABP Organic Anion Binding Protein; PXMP Peroxisomal Membrane Protein; PXMPL PXMP-like; RNASELI RNase L inhibitor; SUR Sulfonylurea Receptor; TAP Transporter Associated with Antigen Processing

3.2 Human ABC Protein Families

In humans, 48 ABC proteins, according to sequence similarity classified into 7 families (A-G), are currently known (Müller, 2002), (Klein, 1999), Figure 7, Table 2).
Besides a general description of each family, some selected human ABC proteins are being referred to in more detail in the next subchapters.

3.2.1 ABCA (ABC1) family

Out of the 12 ABCA family members, four are assumed to transport lipophilic substrates (ABCA1: phospholipids, cholesterol, ABCA2: estramustine (a sterol derivative), ABCA4: N-Retinylidene-PE (a phospholipid derivative), ABCA7: presumably lipids (Kaminski, 2000)) (Müller, 2002). The substrates of the other ABCA members are not known.

ABCA1 (ABC1) is found in various tissues and mediates secretion of sterols and phospholipids into apolipoproteins. Mutations in ABCA1 lead to degradation of apolipoproteins and accumulation of cholesterol in macrophages, increasing the risk of arteriosklerosis (Tangier disease) (Brooks-Wilson, 1999). Furthermore, ABCA1 has been implicated with PS exposure in apoptotic cells and phagocytizing macrophages (Marguet, et al., 1999).

ABCA4 (ABCR), localized in the rods of the retina, is involved in the dark-adaptation through transport of N-Retinylidene-PE. Defective ABCA4 may cause degeneration of the macula lutea and consequent deterioration of vision (Stargardt syndrome) (Allikmets and Lewis, 1997).

3.2.2 ABCB (MDR/TAP) family

The members of the ABCB family show highly varied specificities (ABCB1: amphiphilic compounds, ABCB2,3,10: peptides, ABCB6,7: iron, ABCB4: PC, ABCB11: bile salts) (Müller, 2002).

ABCB1 (designated Multidrug Resistance 1 P-glycoprotein (MDR1 Pgp) in the following) (Juliano, 1976) is a 170 kDa transporter with a surprisingly broad spectrum of amphiphilic substrates, which were reported to be mainly cationic or electrically neutral (Ford, 1990). It occurs in the apical membrane domain (Thiebaut, 1987) of epithelia with secretory functions (e.g. adrenal gland, kidney) and at the pharmacological borders of the body (intestine, blood-brain barrier, feto-maternal barrier) (Borst, 1993). In addition, MDR1 Pgp is found in many tumor tissues (Cordon-Cardo, 1990).
One important physiological role of MDR1 Pgp appears to be the protection of the organism against toxins, achieved by exporting these compounds from the body, e.g. into the bile, urine, or gut.
In tumors, MDR1 Pgp is one of the proteins principally responsible for both intrinsic and acquired multidrug resistance (MDR) against a whole bandwidth of structurally unrelated toxins (*typical MDR* mediated by MDR1 Pgp, versus *atypical MDR* mediated by other factors). This is of major clinical relevance, as multidrug resistance is the main limitation for systemic antitumor chemotherapy, occurring in about 90% of all metastasizing tumors treated with cytostatic drugs (Gottesman, 1993). Resistance can be acquired by the selection of resistant cells from a cell population. Additionally, the drugs used in the therapy may induce the synthesis of MDR proteins (see below). Among others, various anthracyclines (e.g. Daunorubicin, Doxorubicin) and plant alkaloids (e.g. Etiposide, Paclitaxel, Teniposide, Vinblastine, Vincristine) are MDR1 Pgp transport substrates. Despite the development of substances inhibiting MDR1 Pgp in vitro, clinical exploitation of these inhibitors has failed so far, mainly because of their high degree of toxicity (Krishna, 2000).

In 1992, MDR1 Pgp has been proposed to regulate or constitute a chloride channel, similar to ABCC7 (CFTR) (Valverde, 1992). However, recent reports have not confirmed this finding (Tominaga, 1995).

Besides detoxification and multidrug resistance, MDR1 Pgp is involved in other phenomena as well, in which, interestingly, lipid transport often seems to be implicated: MDR1 Pgp was found to mediate secretion of the steroid aldosterone by the adrenals (Bello-Reuss, 2000) and to facilitate the migration of dendritic immune cells (Randolph, 1998), possibly related to outward transport of platelet activating factor (PAF).

Ueda et al. reported MDR1 Pgp mediated transport of the steroids cortisol and dexamethasone, but not of progesterone (Ueda, 1992).

In the past years, several works have dealt with the transport of glycerophospholipids, sphingolipids and their analogs by MDR1 Pgp (Table 3):

Table 3: Lipids and lipid analogs transported by MDR1 Pgp.

Lipid	Analog	Reference
PC	C6-NBD	(van Helvoort, 1996)
	C8-C8	(van Helvoort, 1996)
	C12-NBD	(Bosch, 1997)
	C16 ether-C2 (PAF)	(Ernest and Bello-Reuss, 1999)
PE	C6-NBD	(van Helvoort, 1996)
	C8-C8	(van Helvoort, 1996)
	C12-NBD	(Bosch, 1997)
PS	~~C12-NBD~~	(Bosch, 1997)
SM	C6-NBD	(van Helvoort, 1996)
GlcCer	C6-NBD	(van Helvoort, 1996)
	C8-C8	(van Helvoort, 1996)
	endogenous?	(Raggers, 2000)

Crossed-out analogs were reported not to be MDR1 Pgp substrates, while analogs followed by an interrogation mark are assumed but not confirmed to be MDR1 Pgp substrates.

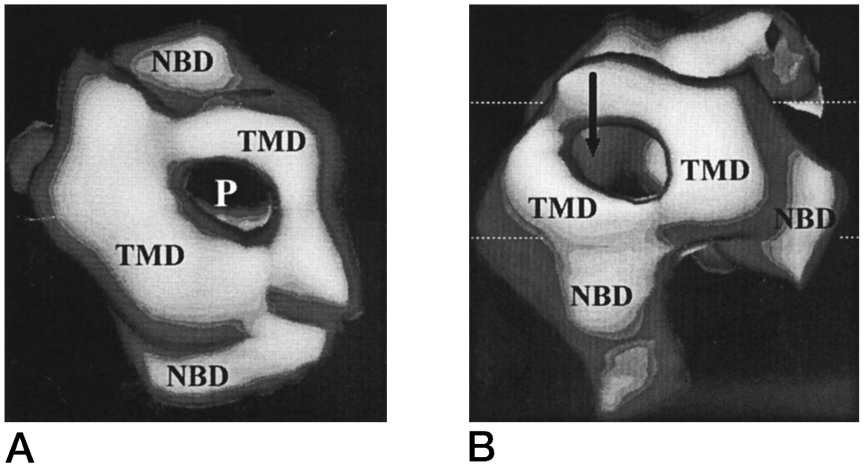

Fig.8: MDR1 Pgp structure

In the 3-D reconstruction images obtained by electron microscopy and image analysis in the absence of ATP, A provides a top view of MDR1 Pgp from the exoplasmic side of the membrane (P: central pore; NBD: nucleotide binding domain (ABC domain); TMD: transmembrane domain),
B gives a lateral view of MDR1 Pgp, the arrow marking a side entrance from the lipid phase into the chamber (taken from (Rosenberg, 1997)).

A variety of short-chain and long-chain lipid analogs were found to be expelled from the cell by MDR1 Pgp (van Helvoort, 1996), (Bosch, 1997), and integration of the substrate into the membrane was suggested to be required for MDR1 Pgp mediated transport (thus acting as an unspecific flippase), rather than specific recognition of a structural motif (Higgins and Gottesman, 1992). Among the endogenous lipids, the short-chain PC PAF (Ernest and Bello-Reuss, 1999) is an MDR1 Pgp substrate and, possibly, glucosylceramide as well (Raggers, 2000).
The MDR1 Pgp mouse homologs Mdr1a/1b were found to be unable to restore transport of PC into the bile of *Mdr2* knockout mice (Smit, 1993). While this could suggest that natural long-chain PC is not an MDR1 Pgp substrate, it is also conceivable that Mdr1a/1b activity was too low in this model. Due to its low substrate specificity, MDR1 Pgp might affect the transverse distribution of endogenous lipids, in particular of species which are normally predominant on the inner plasma membrane leaflet, such as PS and PE. Unlike other MDR1 Pgp substrates, PS is anionic.

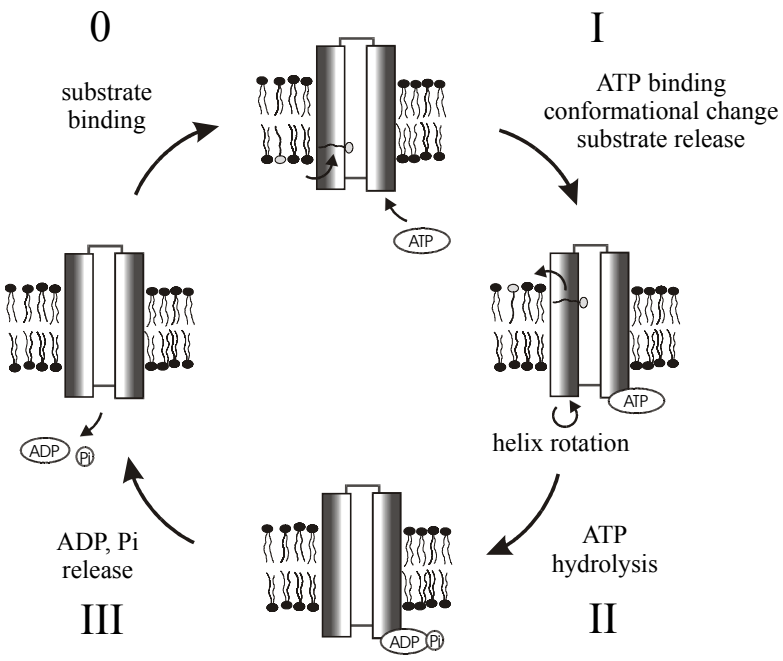

Fig.9: Rotating helix flippase model of substrate transport by MDR1 Pgp.

Via the transmembrane domain interfaces of the transport protein, the substrate (shown in grey) enters the central chamber laterally from the cytoplasmic membrane leaflet (0). Subsequent to ATP binding, a helix rotation induces substrate release on the exoplasmic membrane leaflet (I). Then, ATP is hydrolyzed and a second conformational change occurs (II). In the ensuing step, ADP and Pi are released and the protein regains its initial, substrate-binding state through a third conformational change (III).

However, its amphiphilic nature might be sufficient for transport via an unspecific flippase. MDR1 Pgp is made up of two halves highly similar in sequence, each consisting of a transmembrane domain made up of six alpha-helices and an ABC domain. MDR1 Pgp is glycosylated at the first exoplasmic loop (Germann, 1996). After synthesis in the ER and modification in the Golgi (found for rat Mdr1b) (Sai, 1999), MDR1 Pgp glycosylation is presumably necessary for its transport to the apical plasma membrane. The functional unit of MDR1 Pgp appears to be a monomer (Loo, 1996).

Electron microscopy structure analyses on MDR1 Pgp have revealed some interesting features of this transport protein (Fig.8): The transmembrane domains enclose a large hydrophilic chamber which opens towards the exoplasmic face of the membrane (Rosenberg, 1997), (Rosenberg, 2001) (e.g. contrasting with the tightly packed transmembrane domains of ion-translocating ATPases) and exhibits side entrances towards the lipid bilayer.

This structure supports the flippase concept for MDR1 Pgp mediated transport, enabling amphiphilic substrates to laterally enter the central chamber from the membrane.
Based on data obtained from MDR1 Pgp trapped at different stages of the ATPase cycle, a model for the transport process has been put forward involving the rotation of transmembrane helices, (Fig.9): The transporter binds the substrate on its cytoplasmic side, binds ATP, releases the substrate on its exoplasmic side, hydrolyzes ATP and releases ATP and Pi (Rosenberg, 2001). The major conformational changes occur in MDR1 Pgp upon ATP binding rather than during ATP hydrolysis or release.
In the literature, numerous mechanisms have been proposed for MDR1 Pgp regulation:
- On the DNA level, amplification (Roninson, 1992) of the *MDR1* gene localized in 7q21.1 has frequently been found in drug-selected cell lines.
- On the transcription level, anthracyclines, but not plant alkaloids, rapidly cause up-regulation of *MDR1* RNA (Hu, 1995) by an unknown mechanism.
Methylation of the *MDR1* promoter region has been reported to reduce *MDR1* transcription (Kusaba, 1999), while p53 inactivation, frequently occurring in cancers, leads to *MDR1* transcription up-regulation (Thottassery, 1997).
- On the protein level, the reports in literature diverge on whether or not phosphorylation of MDR1 Pgp has an effect on its activity (Szabo, 1997), (Castro, 1999), (Germann, 1996). In addition to the above mentioned, a diversity of further potential MDR1 Pgp regulators have been reported (Kantharidis, 2000).

ABCB2 and *3* (TAP1 and 2) are involved in the immunorecognition process. A heterodimer made up of ABCB2 and 3 transports proteasome-cleaved peptides into the ER lumen, where the peptides associate with Major Histocompatibility Complex (MHC) class 1 molecules. Later, these peptides can be presented on the cell surface for recognition by lymphocytes (Klein, 1999).

ABCB4 (designated MDR2/3 Pgp in the following) is a close relative of MDR1 Pgp, sharing 75% of its amino acid sequence. Unlike MDR1 Pgp, MDR2/3 Pgp is highly specific, exclusively transporting PC and its analogs (van Helvoort, 1996). The physiological function of MDR2/3 Pgp appears to be PC secretion into the bile (Smit, 1993), MDR2/3 Pgp being present in high amounts in the canalicular membranes of hepatocytes (Smith, 1994). In some cases of progressive familiar intrahepatic cholestasis, MDR2/3 Pgp has indeed been found to be defective (Deleuze, 1996).

3.2.3 ABCC (CFTR/MRP) family

Major functions of ABCC proteins are, among others, the protection against toxic compounds and the secretion of organic anions (ABCC1,2,3: inorganic conjugates, ABCC3: bile salts, ABCC4,5: nucleotide analogs, ABCC5, 7: organic anions) (Müller, 2002).

ABCC1 (designated MRP1 in the following) (Cole, 1992), located in the basolateral domain (Evers, 1996) of epithelia of the testis and the lung and in peripheral blood mononuclear cells, transports a wide spectrum of toxins across the plasma membrane, either unconjugated or conjugated with glutathione, sulfate or glucuronosyl. MRP1 protects particularly sensitive organs by expelling toxins into the blood (the internal environment, in contrast to the apically located MDR1 Pgp which exports toxins into the external environment). Additionally, MRP1 mediates the leukotriene C (LTC) dependent inflammatory response by transport of the arachidonic acid derivative LTC4. Recently, MRP1 has been reported to transport the lipid analogs C6-NBD-SM, C6-NBD-GlcCer (Raggers, 1999), C6-NBD-PS, C6-NBD-PC and C12-NBD-PC (Kamp, 1998). However, thus far, no endogenous lipids have been found to be MRP1 substrates.

ABCC7 (CFTR) forms a cAMP induced chloride channel in epithelia and is involved in the regulation of other transport pathways. According to the channel definition, substrates pass through the ABCC7 channel in a non-stochiometric manner, following their concentration and electrochemical gradient. However, ATP is thought to play a gating role for this passage. ABCC7 mutation leads to the common hereditary disease cystic fibrosis (mucoviscidosis), characterized by a disruption of pancreas exocrine function, by biliary cirrhosis, chronic bronchopulmonary infection, high electrolyte sweat and infertility (Klein, 1999).

3.2.4 ABCD (ALD) family

All known members of the ABCD family have been implicated with the transport of fatty acids (ABCD1: very long chain fatty acids, ABCD2,3,4: fatty acids).

ABCD1, a peroxisomally localized protein, transports very long chain fatty acids into the peroxisome for oxidation. ABCD1 defects can result in neuron demyelinization and renal insufficiency (Adrenoleukodystrophy) (Mosser, 1993).

3.2.5 ABCE (OABP) family

ABCE1 is the unique member of the ABCE family. It exhibits an unusual domain organization, consisting of two ABC domains and completely lacking transmembrane domains. ABCE1 inhibits RNase L by acting as an antagonist to the RNA degradation pathway 2-5A (Klein, 1999).

3.2.6 ABCF (GCN20) family

Like ABCE, ABCF protein family members consist of two ABC domains and lack transmembrane domains. Their functions and substrates are not known (Klein, 1999).

3.2.7 ABCG (WHITE) family

A number of ABCG proteins are thought to be involved in the transport of sterols (ABCG1,5,8), additionally, some members appear to transport phospholipids (ABCG1) and toxins (ABCG2) (Müller, 2002).

ABCG1 (WHITE) derives its trivial name from its homology to the drosophila white protein, which transports guanine and tryptophane as precursors for eye pigments. ABCG1 itself appears to serve a different function, presumably in the transport of phospholipids and sterols out of macrophages (Klucken, 2000).

ABCG2 (designated BCRP in the following) (Allikmets, 1998), detected in the placenta, intestinal epithelium, liver canaliculi, breast ducts and lobules, as well as in veinous and capillary endothelium (Maliepaard, 2001), is a 72 kDa transport protein consisting of 655 amino acids. It is assumed to prevent the uptake of xenobiotics into these tissues (Maliepaard, 2001). In an ATP dependent process, BCRP transports various xenobiotics and cytostatic drugs across the plasma membrane, e.g. the anthracenes mitoxantrone and bisantrene, several anthracyclines, and the camptothecin derivative topotecan (Litman, 2000). Transfection studies proved overexpression of *BCRP* to induce multidrug resistance in a previously drug sensitive cell line (Doyle, 1998). As BCRP contains only one ABC domain and a transmembrane domain presumably made up by 6 alpha-helices, this so-called half-size transporter is thought to homo- or heterodimerize to form an active transport complex in the plasma membrane (Bates, 2001). In addition to drug transport, recent works have given hints on the transport of lipid analogs by BCRP (Litman, 2000).

Both *ABCG5* (WHITE3) and *ABCG8* (WHITE4) are localized in liver and intestine. Experimental data links them to cholesterol transport. Defects in these two proteins can result in increased uptake of cholesterol in the intestine, combined with decreased bilary excretion, causing arteriosklerosis (Sitosterolemia) (Berge, 2000).

4 Aim

In eukaryotic cells, lipids can be transported actively between the membrane leaflets. However, only a small number of lipid transport proteins has been identified until today. Members of the ABC protein superfamily have recently been implicated with the transport of lipids, e.g. MDR1 Pgp, a full-size transporter which mediates multidrug resistance (MDR) in tumors by active outward transport of various cytostatic drugs (Germann, 1996). Despite its high clinical relevance and profound characterization, attempts to inhibit MDR1 Pgp in vivo have not been successful so far.
Like MDR1 Pgp, the half-size transporter BCRP is a multidrug resistance protein (Doyle, 1998). BCRP has been characterized only in part, and transport of a lipid analog by BCRP has merely been shown indirectly (Litman, 2000).

Does MDR1 Pgp transport zwitterionic and neutral lipid analogs
in MDR1 overexpressing human gastric carcinoma cells?
MDR1 Pgp was reported to transport analogs of the lipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and glucosylceramide (GlcCer) (van Helvoort, 1996), which are used here to characterize the lipid transport activity of MDR1 Pgp in a human gastric carcinoma cell line. In addition, the part that MDR1 Pgp plays in the accumulation of analogs of diacylglycerol (DG) and ceramide (Cer) is regarded. These lipids move across the membrane comparatively fast due to their small, uncharged headgroup (Zachowski, 1993), which could make them interesting competitors of MDR1 Pgp mediated drug transport. DG, Cer, SM and GlcCer are furthermore involved in the SM signalling pathway (Perry, 1998).

Does MDR1 Pgp transport the anionic lipid PS?

While MDR1 Pgp has been reported to transport mainly cationic or electrically neutral substrates (Ford, 1990), the flippase mechanism suggested for MDR1 Pgp mediated transport from the inner to the outer membrane leaflet predicts integration of the substrate into the membrane, rather than recognition of a particular structural motif, to be required for transport (Higgins and Gottesman, 1992). In this case, MDR1 Pgp might also transport anionic lipids such as PS, the lipid generally exhibiting the most pronounced transverse asymmetry in the plasma membrane (Zachowski, 1993). Yet, the inward transporting aminophospholipid translocase activity might counteract PS outward transport. PS exposure is linked to cell activation (Bevers, 1982) and apoptosis (Fadok, 1992) and has been observed in some tumorigenic cell lines (Utsugi, 1991). In the human gastric carcinoma cell line used above, a new labelling assay is set up to study the role of MDR1 Pgp in the outward transport of a fluorescent analog of PS. While the outward transport of endogenous PS cannot be assessed directly, the exposure of endogenous PS on the outer leaflet of the plasma membrane is determined here. Further experiments in four cell lines of different species and tissue origin (canine and porcine epithelial cells and human and murine fibroblasts) allow comparison of PS outward transport in models with divergent transport backgrounds and varying synthesis levels of MDR1 Pgp.

Does the multidrug resistance protein BCRP transport lipids?

BCRP has been suggested to decrease accumulation of a ceramide analog (Litman, 2000). In human gastric carcinoma cells, outward transport of a PS analog and exposure of endogenous PS on the outer leaflet of the plasma membrane are studied to start to characterize the substrate profile of BCRP, and to prognosticate whether low-specificity transport of lipid substrates might be a frequent feature of MDR proteins.

5 Materials and Methods

5.1 Materials

Brefeldin A (BFA), Diisopropyl fluorophosphate (DFP), fatty acid-free bovine serum albumin (BSA), FITC-Concanavalin A (FITC-ConA), Hepes and Rhodamine 123 (Rho 123) were purchased from Sigma-Aldrich (Steinheim, Germany). Leibovitz L15 medium was obtained from Bio Whittaker (Walkersville, MD, USA), fetal calf serum and M199 medium were from GIBCO/BRL (Grand Island, NY, USA). DMEM, DMEM/F 12, cell culture supplements, chambered coverglasses (Nunc) and Dulbecco's phosphate buffered saline with Ca^{2+}, Mg^{2+}(PBS) were obtained from Biochrom KG (Berlin, Germany), for mPBS, PBS was supplemented with 24 mM glucose and 10 mM Hepes. C6-NBD-ceramide (-Cer), -phosphatidic acid (-PA), -phosphatidylcholine (-PC), and -phosphatidylserine (-PS) were purchased from Avanti Polar Lipids (Birmingham, AL, USA), high performance thin layer chromatography (TLC) plates from Merck (Darmstadt, Germany). FITC-Annexin V, binding buffer and propidium iodide were bought from VPS diagnostics (Hoeven, The Netherlands), CellTracker Green (5-chloromethyl-fluoresceine diacetate, CMFDA), MitoTracker Red CMXRos and TRX-Wheat germ agglutinin (WGA) from Molecular Probes (Leiden, The Netherlands). Transwell filters with 4.7 cm^2 diameter, 0.4 μm pore size were bought from Costar (Cambridge, MA) and Triton X-100 from Fluka (Buchs, Switzerland). Inhibitors of ABC transporters used: Cyclosporin A and glyburide (Sigma-Aldrich, Steinheim, Germany), PSC 833 (Novartis, Basel, Switzerland), dexniguldipine-HCl (B8509-035, Byk Gulden, Konstanz, Germany), and MK 571 (Merck-Frosst, Pointe-Claire-Dorval, Canada), tryprostatin A (H. Osada, Riken Institute, Japan).

5.2 Cells

The human gastric carcinoma cell line EPG85-257 is derived from human gastric epithelial tumor tissue (Dietel, 1990). By 2 to 24 month selection of parental drug-sensitive EPG85-257P cells (EPG85-257 control line) with increasing concentrations of daunorubicin (for *MDR1* overexpression) or mitoxantrone (for *BCRP* overexpression) (Lage, 2000), the multidrug resistant sublines EPG85-257RDB (EPG85-257 *MDR1* overexpressing line) and EPG85-257RN (EPG85-257 *BCRP* overexpressing line) were obtained. EPG85-257 cell lines were provided by Dr. H. Lage (Charité, Institute of Pathology,Humboldt University Berlin).

LLC-PK1 cells originate from normal pig kidney epithelial tissue (Hull, 1976). Transfection with human *MDR1* yielded the LLC-PK1 *MDR1* overexpressing subline (Schinkel, 1995).

The murine fibroblast cell line MF is derived from normal, Mdr1a/b$^{+/+}$ MRP^{+} wildtype (WT12) mice, while the mouse ear fibroblast knock-out (KO) cell line MF Mdr1a/b$^{-/-}$ MRP^{-} is derived from Mdr1a/b$^{-/-}$ MRP^{-} knock-out mice (Allen, 1999) with a mixed genetic background of 50% FVB, 50% 129/OLA. LLC-PK1 and MF cell lines were used in the lab of Prof. G. van Meer (Department of Membrane Enzymology, University of Utrecht, The Netherlands).

The Madin-Darby canine kidney cell line MDCKII was obtained from normal dog kidney epithelial tissue (Madin, 1958) and was kindly provided by Prof. G. van Meer (Department of Membrane Enzymology, University of Utrecht, The Netherlands).

Human primary gingival fibroblasts (KPG7) were obtained from young healthy male volunteers (Pomorski, 1996).

5.3 Cell Culture

EPG85-257 cells were cultured in Leibovitz L15 medium supplemented with 10% FCS, 1 mM L-glutamine, 6,25 mg/l fetuin, 80 IE/l insulin, 2,5 mg/l transferrin, 1 g/l glucose, 1,1 g/l $NaHCO_3$, 1% minimal essential vitamins and 20 000 kIE/l trasylol.
EPG85-257RDB cells were cultured in the presence of 2.5 µg/ml daunorubicin, and EPG85-257RN cells in the presence of 0.2 µg/ml mitoxantrone. Cells were grown in the absence of cytostatic drugs for 96 hours prior to experiments.
DMEM medium supplemented with 10% FCS was used for the culture of both MDCKII and MF cells. For MDCKII cells, medium additionally contained 1% penicillin/streptomycin and 1% sodium pyruvate.
LLC-PK1 cells were grown in M199 medium supplemented with 10% FCS.
KPG7 human fibroblasts were grown in DMEM/F 12 medium supplemented with 10% FCS, 2 mM glutamine, 50 mg/l ascorbic acid and antibiotics/antimycotics (105 units/l of penicillin,100 mg/l of streptomycin, 2.5 mg/l amphotericin B).
For C6-NBD-Cer labelling, LLC-PK1 cells were grown on polycarbonate filters. All other cells were grown on 35 mm culture dishes for spectroscopy, and on two-chamber coverglasses for microscopy. Viability was determined at the end of the experiments by trypan blue exclusion (final concentration 0.5%) in aliquots. Non-viable cells did not exceed 3% even when cells were pretreated with inhibitors.

5.4 Inhibitors and BFA

Cyclosporin A, glyburide, BFA and PSC 833 stocks were prepared in ethanol, MK 571 was prepared in double-distilled water, dexniguldipine-HCl in dimethyl sulfoxide and tryprostatin A in methanol. For all experiments, the effect of the solvent was determined. Final concentrations unless otherwise indicated: cyclosporin A: 25 µM, glyburide: 200 µM; PSC 833: 10 µM; MK 571: 25 µM, dexniguldipine-HCl: 20 µM; BFA: 5 µM; tryprostatin A 5 µM.

5.5 Spectroscopy Transport Assays

Measurement of Rho 123 and GS-MF outward transport

Cells preincubated with or without MDR inhibitors for 10 minutes on ice were loaded for 15 minutes at 37°C with the fluorescent probes 10 µM Rho 123 or 3.2 µM CMFDA, washed with ice-cold mPBS, and incubated for 10 minutes on ice in mPBS with or without MDR inhibitors. Assays were started by transferring the dishes to 37°C. At given time points, media were collected, and cells scraped into mPBS. Fluorescence of media and scraped cells was measured in the presence of 0.5% Triton X-100 at excitation/emission wavelengths of 470 nm/540 nm and 4 nm/8 nm slit width for Rho 123 and GS-MF, using an Aminco Bowman Series 2 fluorescence spectrometer (Urbana, IL).

Measurement of C6-NBD-PC, -PE, GlcCer, SM and -Cer outward transport

Transport of newly synthesized C6-NBD-PC, -PE, -GlcCer and -SM to the exoplasmic leaflet of the plasma membrane was assessed at 15°C as described by van Helvoort et al. (van Helvoort, 1996). In addition, accumulation of -DG and -Cer was assessed. Cells preincubated for 10 minutes in the presence or absence of MDR inhibitors or the antibiotic BFA were incubated at 15°C with 25 µM C6-NBD-PA (for -PC and -PE synthesis) or 5 µM C6-NBD-Cer (for -DG, -SM and -GlcCer synthesis) in 1% w/v BSA in mPBS with or without MDR inhibitors or BFA. After 180 minutes, BSA-containing media were collected, and cells subjected to a 30 minute back exchange incubation on ice with BSA in mPBS, followed by lipid analysis of cells and combined media.

For polarized LLC-PK1 cells grown on polycarbonate filters, the above protocol was modified: Cells were labelled with lipid analogs from both the upper (apical) and the lower (basolateral) face of the filters. Subsequent to BSA extraction following the 15°C incubation, filters were cut from the supports, and lipids extracted from filters, apical and basolateral media.

Measurement of C6-NBD-PS and -PC inward transport.

After a 10 minute preincubation with or without MDR inhibitors, cells were labelled with 10 µM C6-NBD-PS or 14 µM C6-NBD-PC in cold mPBS (Pomorski, 1996). After a 15 minute incubation on ice, non-inserted analogs were removed by washing with cold mPBS. About 230 pmol NBD lipid analog was inserted per dish (1.5 x 10^6 cells). Kinetic measurements were started by addition of 20°C mPBS (with or without MDR inhibitors), containing 5 mM DFP (as all following incubations) to prevent hydrolysis of labelled phospholipid (Colleau, 1991). At given time points, culture dishes were transferred on ice and BSA (final 2% w/v) was added to extract NBD lipid from the cell surface during a 10 minute incubation. Cells were again subjected to a 10 minute back exchange on ice with BSA in cold mPBS. BSA-containing media were collected and lipids of cells and combined media were analyzed.

Measurement of C6-NBD-PS outward transport

Cells were preincubated with or without MDR inhibitors for 10 minutes, labelled with 5 to 10 µM C6-NBD-PS for 15 minutes on ice, and incubated at 20°C for 30 minutes to allow inward movement of the NBD analog. C6-NBD-PS remaining on the cell surface was extracted twice by incubation with 2% (w/v) BSA in mPBS for 10 minutes on ice. Before starting the outward transport assay, the medium was removed, and cells were washed with cold mPBS. For t=0', cold medium (2% (w/v) BSA and 5 mM DFP in mPBS with or without inhibitor) was immediately added to the cell dish and incubated for 10 minutes. To measure C6-NBD-PS outward transport, prewarmed (15°C or 37°C) mPBS with 2% (w/v) BSA and 5 mM DFP was added to the dish, and cells were incubated at 15°C or 37°C for indicated lengths of time. Removal of the BSA-containing media and a second extraction with 2% BSA in mPBS for 10 minutes on ice terminated incubations, followed by lipid analysis of cells and combined media.

5.6 Lipid Analysis

5.6.1 Lipid extraction

Subsequent to the incubations, the BSA back exchange media were combined, and cells were scraped into mPBS. For C6-NBD-PS analysis, lipids from both scraped cells and media were extracted with isopropanol (5.5 ml per 1ml of probe) to prevent substantial loss into the aqueous phase. Samples were centrifuged at 780 *g* for 5 minutes, the supernatant transferred into new glass tubes and dried.

For extraction of all other C6-NBD lipid analogs, a method adapted from Bligh and Dyer (Bligh, 1959) was used for lipids from both cells and incubation media: 3.2 ml methanol/chloroform (2.2:1, v/v) were added to 1 ml of probe, and incubated for 30 minutes at room temperature. 1 ml 20 mM acetic acid and 1 ml chloroform were added to the solvent mixture, followed by a 10 minute centrifugation at 800 *g*. While the resulting lower organic phase was secured, 1 ml chloroform was added to the upper aqueous phase which was again centrifuged, and the new lower phase combined with the first.

5.6.2 Lipid separation

Probes dried under nitrogen were applied on $60F_{254}$ silica TLC plates with 2x5 drops of chloroform/methanol (1:1, v/v). After 2-D separation [I, chloroform/methanol/25% ammonium hydroxide (13:5:1, v/v); II, chloroform/acetone/methanol/18 M acetic acid/water (6:8:2:2:1, v/v)], fluorescent lipid spots were visualized under ultraviolet light and individually scraped off the plates.

5.6.3 Lipid quantification

C6-NBD-lipid was extracted from silica with 2 ml chloroform/methanol (1:1, v/v). After silica sedimentation, C6-NBD-lipid was quantified spectroscopically (470 nm excitation, 540 nm emission wavelength) by reference to C6-NBD-lipid standards.

For experiments on LLC-PK1 and MF cells, TLC plates were scanned with the gel and blot imaging system Storm (Molecular Dynamics) and analyzed via Imagequant software. Below, C6-NBD-lipid in the BSA medium (derived from the exoplasmic leaflet of the plasma membrane and residual labelling medium) is termed extractable, the amount remaining in the cells (in the cytoplasmic leaflet of the plasma membrane and the cell interior) non-extractable. For experiments on polarized cells, only the apical and not the basolateral medium is regarded.

5.7 Microscopy Transport and Labelling Assays

Measurement of Rho 123 accumulation

Cells were incubated in mPBS with or without inhibitors for 10 minutes at 37°C. Loading of cells with 2 - 5 µM Rho 123 in mPBS occurred during a 60 minute incubation at 37°C in the presence or absence of the respective inhibitors. After washing, green Rho 123 fluorescence of cells was observed by microscopy.

Measurement of GS-MF outward transport

Cells were pretreated with inhibitors as described for the accumulation of Rho 123. They were loaded with 2.5 µM CMFDA in mPBS for 15 minutes at 37°C and washed. CMFDA is cleaved intracellularly by esterases and subsequently forms the glutathione conjugate GS-MF. For inverse fluorescence microscopy, cells were incubated with mPBS for 30 minutes at 37°C in the presence or absence of the inhibitor MK 571, and washed prior to examination of green GS-MF fluorescence.

Measurement of C6-NBD-PS outward transport

Cell plasma membranes were labelled with 0.5 - 2 µM C6-NBD-PS for 20 minutes on ice, then the analog was allowed to accumulate intracellularly during a 30 minute incubation at 20°C in the presence or absence of inhibitors used.

After extraction of analog remaining on the cell surface, cells were treated as described for C6-NBD-PS outward transport in 5.5. Before microscopic examination, BSA was removed and cells were washed twice.

Labelling with MitoTracker

To obtain mitochondrial labelling (Poot, 1996), cells were incubated with 30 nM MitoTracker Red CMXRos in mPBS at 37°C for 30 minutes. After washing, red CMXRos fluorescence was examined by microscopy.

Labelling with C6-NBD-Cer and its metabolic products

For localization studies of C6-NBD-Cer and its metabolic products, cells preincubated at 37°C in mPBS in the presence or the absence of BFA for 20 minutes, were labelled for 10 minutes on ice with 2 - 5 µM C6-NBD-Cer in mPBS containing 0.1% BSA (w/v) (Lipsky, 1985), and incubated at 37°C with mPBS in the presence or the absence of BFA for 60 minutes. Before washing and microscopic observation, cells were twice subjected to a 10 minute back exchange on ice with 2% (w/v) BSA in mPBS.

Labelling with TRX-WGA

Cells were fixed during a 15 minute incubation with 3% formaldehyde in PBS at room temperature, and permeabilized at room temperature with 0.1% Triton X-100 in PBS for 5 minutes. Subsequently, cells were washed and labelled with 1 µM TRX-WGA in PBS during a 20 minute incubation at 37°C. Prior to microscopy, cells were washed.

Labelling with FITC-ConA

Cells were fixed, permeabilized and washed as described above. Labelling with 2 µM FITC-ConA in PBS was performed during a 20 minute incubation at 37°C. Fixing was repeated, and cells were observed microscopically after washing.

Fluorescence Microscopy and Photography

Microscopy was performed with an inverse standard microscope equipped with a Plan-Neofluar 100x (1.3 NA) objective. The barrier filter sets (Carl Zeiss, Oberkochen, Germany) used were: green fluorescence: BP 450 to 490 excitation filter, FT 510 dichroic mirror and LP 515 emission filter; red fluorescence: BP 546 excitation filter, FT 580 dichroic mirror and LP 590 emission filter. Photographs were taken using Kodak EPH P 1600 X films push-processed to 3200 ASA. As fluorescence photobleached quickly, different cells were photographed after each treatment.

5.8 Annexin Assay

To measure exposure of endogenous PS on the cell surface, cells were incubated on ice for 10 minutes in the dark with 9 nM FITC-Annexin V in binding buffer with or without PSC 833, following a 30 minute incubation with or without the inhibitor at 37°C. Two minutes prior to the end of the incubation, propidium iodide (final 1.9 µM) was added. Cells were then washed three times, detached from the dishes through pipetting, and suspended in binding buffer. Measurement was performed with a FACSCalibur flow cytometer (Becton Dickinson, St. Louis, MO) 5 minutes after the end of incubation. Forward scatter was set on a linear scale with the voltage set E00 and gain 1.39, side scatter on a linear scale with 386 V and gain 1. FITC fluorescence channel was set on a log scale with 646 V, gain 1, and 0.9% compensation of the propidium iodide signal. Propidium iodide fluorescence channel was set on a log scale with 621 V, gain 1, and 24.9% compensation of the FITC signal. Data were analyzed by Becton Dickinson CellQuest software.

5.9 Statistical Analysis

Results are presented as mean +/- standard error (S.E.M.) and were analyzed statistically using a 2-way analysis of variance (ANOVA) Tukey test (Jandel SigmaStat 2.0) considering treatment and day of experiment. Differences were considered significant for $p < 0.05$.

6 Lipid Transport via MDR1 Pgp in EPG85-257 Human Gastric Carcinoma Cells*

In this chapter, MDR1 Pgp mediated outward transport of C6-NBD-lipids is studied in the human gastric carcinoma cell line EPG85-257P (control line, chapter 5.2) and its multidrug resistant subline EPG85-257RDB (*MDR1* overexpressing line, chapter 5.2). The cell lines are characterized concerning their organelle structure and MDR1 Pgp and MRP1 activities.
In 1996, van Helvoort et al. described the ABC protein MDR1 Pgp to be a lipid translocase of broad specificity, C6-NBD-PC, -PE, -SM and -GlcCer being MDR1 Pgp substrates (van Helvoort, 1996). As transport of lipid analogs appears to be a property shared by other ABC proteins, e.g. MRP1 (Dekkers, 1998) or ABCA1 (Hamon, 2000), the use of inhibitors for different ABC proteins allows identification of the respective transporter.
In two biosynthesis experiments, outward transport of C6-NBD-PC, -PE (precursor: C6-NBD-PA), respectively outward transport of C6-NBD-SM, -GlcCer (precursor: C6-NBD-Cer) and accumulation of -DG and -Cer is assessed in EPG85-257 cells, and the intracellular localization of C6-NBD-Cer and its metabolic products are determined by microscopy. Differences in the accumulation of C6-NBD-DG and -Cer, as well as in the synthesis of the metabolic products of C6-NBD-PA, -DG, and -Cer, provide information on whether these lipid analogs exhibiting fast passive flip-flop are MDR1 Pgp substrates.
Like C6-NBD-PC, -PE, -SM and -GlcCer, many MDR1 Pgp substrates are cationic or electrically neutral amphiphiles, and in 1997, Bosch et al. reported the anionic C12-NBD-PS to be no MDR1 Pgp substrate.
Here, outward transport of C6-NBD-PS is studied in EPG85-257 cells.

* Part of this chapter will be published in Biochemical Journal later this year (Pohl, A., Lage, H., Müller, P., Pomorski, T., Herrmann, A. (2002): Transport of phosphatidylserine via MDR1 P-glycoprotein in a human gastric carcinoma cell line, Biochemical Journal 365 [1], pp. 259-268.).

However, metabolic conversion of C6-NBD-PA to C6-NBD-PS cannot be obtained for intracellular labelling with C6-NBD-PS.
To check feasibility of intracellular labelling using the aminophospholipid translocase import activity, C6-NBD-PS (an aminophospholipid translocase substrate) and C6-NBD-PC (not a substrate) inward transport is compared, and the intracellular localization of C6-NBD-PS is determined.
The C6-NBD-lipids employed here are commonly used as analogs for endogenous lipids, but differ from the respective natural lipids in their short fatty acid chain in the C2 position and the attached fluorescent group. The PS binding protein conjugate FITC-Annexin V permits detection of endogenous PS exposed on the outer plasma membrane leaflet and is used to evaluate PS exposure in *MDR1* overexpressing and control EPG85-257 cells.

6.1 EPG85-257 Human Gastric Carcinoma Cells

Human gastric carcinoma EPG85-257P cells (control) show only low synthesis of MDR1 Pgp (Stein, 2002), (Western Blot, Fig.10) and are sensitive to cytostatic drugs (Lage, 2000). The subline EPG85-257RDB (*MDR1* overexpressing) shows elevated synthesis of MDR1 Pgp (Fig.10) (about 10-fold higher than in controls (Stein, 2002)). EPG85-257RDB cells tolerate e.g. 1800-fold higher concentrations of Daunorubicin than controls (Lage, 2000). MRP1 synthesis is only slightly higher in *MDR1* overexpressing cells than in control EPG85-257 cells (Fig.10) (1.2-2-fold higher than in controls (Stein, 2002)). Expression of *ABCA1* is low in both sublines, modulated only very slightly in *MDR1* overexpressing cells, as indicated by RT-PCR analysis (Fig.10).

6.2 Organelle Labelling

EPG85-257 cells were stained with different probes which specifically label cellullar organelles, including MitoTracker CMXRos (mitochondrial marker) and the fluorescent lectine conjugates TRX-WGA and FITC-ConA (Golgi and ER markers). Organelle staining was used to identify C6-NBD-lipid labelled intracellular structures.

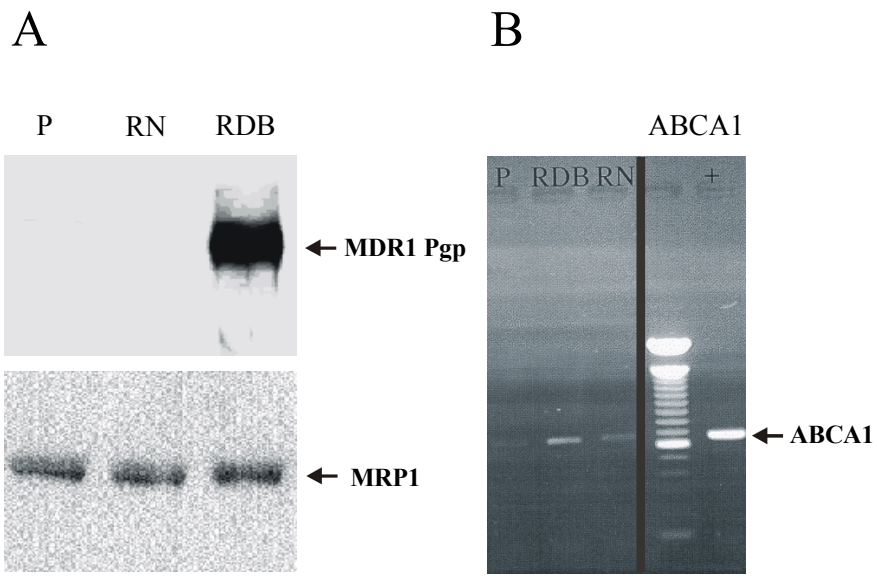

Fig.10: Synthesis of MDR1 Pgp and MRP1 and expression of *ABCA1* in EPG85-257 cells.

A: Western Blot using C219 mouse mAb directed against human MDR1 Pgp and MRPm6 mouse mAb directed against MRP1. The secondary antibody was peroxidase-conjugated rabbit anti-mouse IgG, visualized by chemoluminescence (taken from (Pohl, 2002)).
B: Reverse transcription-PCR for the ABCA1 amplicon (1% agarose gel, ethidium bromide staining), positive control (+), (kindly provided by Dr. D. Kerbiriou-Nabias and Dr. I. Laude, INSERM, Le Kremlin-Bicetre, France). The EPG85-257 sublines tested were EPG85-257P (control), EPG85-257RN (*BCRP* overexpressing), and EPG85-257RDB (*MDR1* overexpressing).

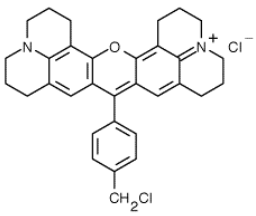

Fig.11: Structural formula of MitoTracker Red CMXRos.

(taken from: (Molecular_Probes, 2002))

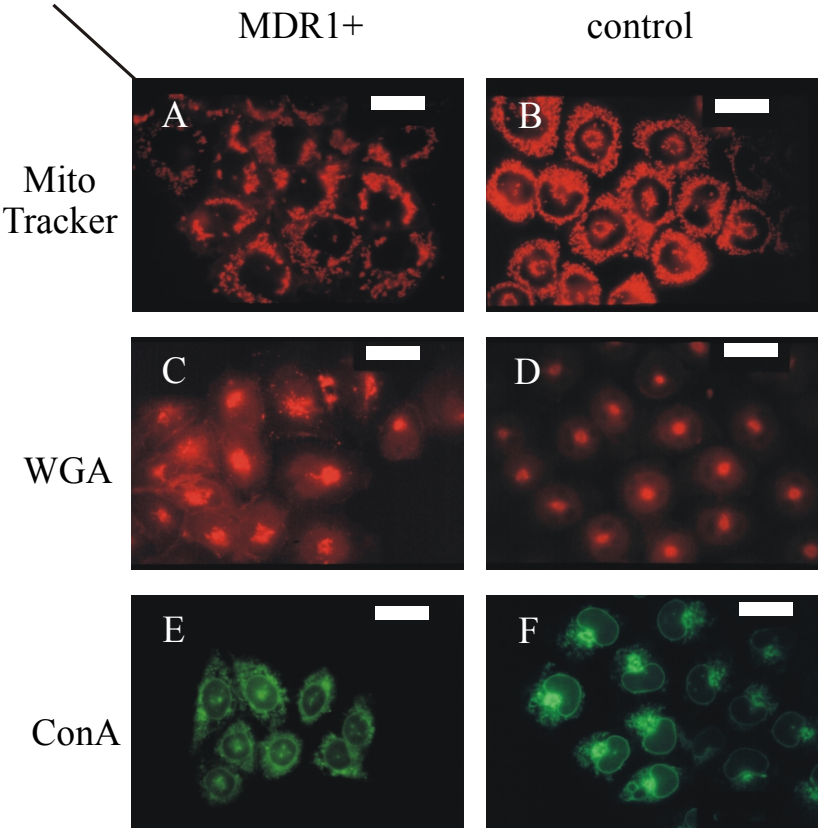

Fig.12: Organelle labelling in EPG85-257 cells, fluorescence microscopy.

Labelling of cells with MitoTracker Red CMXRos (A, B), TRX-WGA (C, D) and FITC-ConA (E, F, taken from different experiments) was performed as described in chapter 5. Labelling concentrations and exposure times are identical for both images with the same organelle label. Bar corresponds to 20 µm.

6.2.1 MitoTracker CMXRos

MitoTracker Red CMXRos (Fig.11) is a X-Rosamine derivative developed for selective staining of mitochondria (Molecular_Probes, 2002). In EPG85-257 cells, labelling with MitoTracker Red CMXRos resulted in distinct fluorescence of granular structures in the cytosol around the nucleus (Fig.12). Particularly in controls, additional fluorescent granuli could be seen organized in a ring surrounding a structure resembling the Golgi (6.2.2), which lay on top of the nucleus. The labelled structures correspond with the description of mitochondria in the literature (Johnson, 1981).

Fluorescence was less distinct in *MDR1* overexpressing cells than in controls, and higher concentrations of MitoTracker Red CMXRos were required to obtain similar fluorescence intensity.
This suggests that MitoTracker Red CMXRos, like Rho 123, might be an MDR1 Pgp transport substrate.

6.2.2 Lectines

Lectines are sugar-binding proteins of mostly botanic origin. Many lectines bind very specifically to particular carbohydrates, e.g. wheat germ agglutinin (WGA) associating with sialic acid and N-acetylglucosaminyl residues, or concanavalin A (ConA), which associates with alpha-mannopyranosyl and alpha-glucopyranosyl residues (Molecular_Probes, 2001). During the biosynthesis of glycoproteins, first N-acetylglucosaminyl, mannosyl and glucosyl residues are added to the nascent polypeptide chain in the lumen of the rough ER.
During further processing in the ER, the glycopolypeptide can be trimmed of its glucose-residues. In the medial Golgi, fucose residues are added, in the trans Golgi galactosyl and sialic acid residues (Kornfeld, 1985).
Fluorescent conjugates of WGA can therefore be used as Golgi markers in permeabilized cells, while fluorescent conjugates of ConA, on the other hand, are used as organelle markers for the rough ER as shown by Virtanen (Virtanen, 1980).

TRX-WGA
Labelling with TRX-WGA was only moderately specific. The cytoplasm exhibited background fluorescence, the nucleus being stained somewhat more intensely, but exhibiting areas of reduced fluorescence intensity (Fig.12). Adjacent to the nucleus, *MDR1* overexpressing cells showed highly intense labelling in scattered structures, while equally strong labelling was seen in a distinct globular region in controls.
WGA has been described to be a marker for the Golgi apparatus. The structures stained brightly by TRX-WGA showed high similarity with the pattern found upon labelling with C6-NBD-Cer and its metabolic products, reported to be lipid Golgi stains (Lipsky, 1985). In both cases, a region adjacent to the nucleus was labelled, appearing compact in controls and more scattered in *MDR1* overexpressing cells.

FITC-ConA

Control cells labelled with FITC-ConA showed staining of the nuclear envelope, the nucleus itself being only weakly fluorescent (Fig.12). Very clearly, fine brightly fluorescent subcellular structures could be seen reaching out from the indentation of the nucleus into the otherwise unlabelled cytoplasm. Fluorescence was most concentrated at the indentation. The plasma membrane was not visible. In *MDR1* overexpressing cells, the nuclear envelope and fine subcellular structures in the cytosol were labelled.
However, there was no or little concentration of FITC-ConA fluorescence at the indentation of the nucleus.
While the Golgi generally concentrates in a narrow space close to the nuclear envelope, the rough ER, reported to be labelled by ConA, tends to invade the cellular cytoplasm further away from the nucleus (David-Pfeuty, 1990). The smooth ER forms tubuli often connected to the Golgi (Novikoff, 1976), and the nuclear envelope is derived from a cistern of the ER (Powell, 2000). FITC-ConA staining overlapped partially with TRX-WGA staining: Both lectines visualized a structure near the indentation of the nucleus in control cells. However, this structure appeared less compact stained with FITC-ConA than with TRX-WGA. This could signify that not the Golgi itself, but smooth ER tubuli connected with the Golgi are labelled by FITC-ConA. Another structure labelled by FITC-ConA was a system of tubuli reaching out from the Golgi region into the cell, corresponding well with the description of the rough ER in literature (David-Pfeuty, 1990). Taken together, the FITC-ConA stained structures in EPG 85-257 cells can be identified as smooth ER, rough ER and the perinuclear cistern of the ER. In contrast to findings in literature, FITC-ConA labels the whole ER system, and not only the rough ER, in EPG85-257 cells.

6.3 Outward Transport of MDR Substrates

The gene products of *MDR1* and *MRP1*, MDR1 Pgp and MRP1, have partially overlapping substrate specificities. However, some substrates have been described to be transported predominantly by one of these two multidrug resistance proteins, allowing determination of the respective activities of either MDR1 Pgp or MRP1. Several MDR inhibitors show a selectivity for either MDR1 Pgp or MRP1.

The *MDR1* overexpressing and the control subline of EPG 85-257 were compared with regards to the respective activities of MDR1 Pgp and MRP1, using the transport substrates Rho 123 and GS-MF, as well as the inhibitors PSC 833, cyclosporin A, dexniguldipine-HCl and MK 571. Microscopy was used to assess the intracellular distribution and homogeneity of labelling in the cell population.

6.3.1 Outward transport and accumulation of Rho 123

Rhodamine 123 (Rho 123) (Fig.13), a tricyclic cationic fluorophore, accumulates in the mitochondria of drug sensitive, but not of multidrug resistant cells after crossing the plasma membrane (Neyfakh, 1988). The reduced accumulation in resistant cells is often due to outward transport of Rho 123 by MDR1 Pgp (Weaver, 1991). In numerous studies, Rho 123 has been used as a model substrate to assess MDR1 Pgp activity in clinical samples (Ludescher, 1992), (van der Kolk, 2001).
Accumulation of Rho 123 per dish (1.5 x 10^6 cells) prior to outward transport was 82 and 206 pmol in MDR1 overexpressing cells and control cells, respectively. When the medium was removed immediately after washing, about 10% of the accumulated Rho 123 was found in the medium. Within 30 minutes at 37°C, significantly higher outward transport of Rho 123 was observed in *MDR1* overexpressing cells than in controls (91% and 49% in the medium, respectively) (Fig.14). In *MDR1* overexpressing cells, all three inhibitors of MDR1 Pgp tested (PSC 833 (Boesch, 1991), cyclosporin A (Cardarelli, 1995), dexniguldipine-HCl (Patterson, 1996)) strongly and significantly decreased Rho 123 outward transport (PSC 833: 50%, cyclosporin A: 58%, dexniguldipine-HCl: 61% of Rho 123 in the medium), whereas the MRP1 inhibitor MK 571 had no significant effect (84% of Rho 123 in the medium). In controls, slight inhibition of the already low Rho 123 outward transport was observed for both PSC 833 (38% of Rho 123 in the medium) and MK 571 (37% of Rho 123 in the medium) (cyclosporin A and dexniguldipine-HCl were not tested).

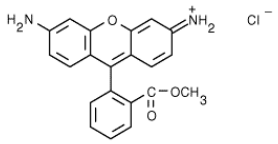

Fig.13: Structural formula of Rhodamine 123.

(taken from (Molecular_Probes, 2001))

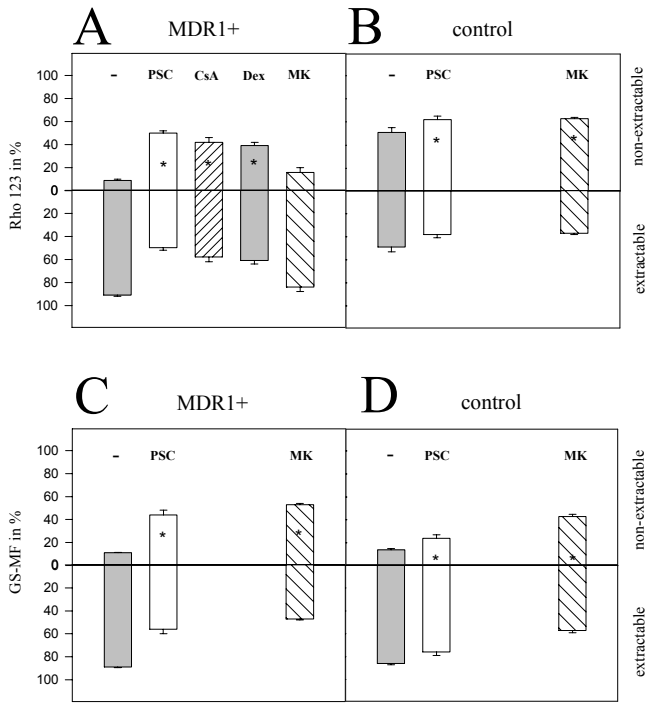

Fig.14: Outward transport of Rho 123 and GS-MF
in *MDR1* overexpressing and control EPG85-257 cells.

MDR1 overexpressing (A, C) and control cells (B, D) were preincubated in the presence or absence of MDR inhibitors PSC 833, cyclosporin A (CsA), dexniguldipine-HCl (Dex), and MK 571. After loading of cells with Rho 123 (A, B) or CMFDA (C, D), outward transport was measured as described in chapter 5. Results are expressed as the percentage of total fluorescence present in the medium after an incubation for 30 minutes at 37°C and represent mean ± S.E.M. of at least n = 3 independent experiments, for MK 571: n = 2; for cyclosporin A and dexniguldipine-HCl: mean ± range of n = 1 experiments with duplicate samples. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) without inhibitor pretreatment ($p<0.05$).

In the literature, MK 571 was reported to be MRP1 specific and not to inhibit MDR1 Pgp (Gekeler, 1995), which is supported by its failure to inhibit Rho 123 outward transport in *MDR1* overexpressing cells. Rho 123 was described to be transported by MRP1, however far less efficiently than by MDR1 Pgp (Minderman, 1996). Inhibition of low, unspecific MRP1 mediated outward transport of Rho 123 could be responsible for the observed inhibitory effect of MK 571 in controls.

Thus, *MDR1* overexpressing cells, but not control cells, exhibit functional (e.g. Rho 123 transporting) MDR1 Pgp in the plasma membrane, corresponding with high synthesis of MDR1 Pgp in the *MDR1* overexpressing subline, and low synthesis in the control line (Pohl, 2002), (chapter 6.1).

In addition to spectroscopy experiments, accumulation and intracellular localization of Rho 123 was assessed by fluorescence microscopy. Low accumulation of Rho 123 in *MDR1* overexpressing cells does not allow microscopic observation of Rho 123 outward transport due to extensive export of Rho 123 occurring already during the labelling process. Therefore, the accumulation of Rho 123 was compared directly in the different sublines in this experiment.

After Rho 123 labelling and washing, control cells displayed throughout strong intracellular fluorescence due to accumulation of Rho 123 (Fig.15). In contrast, *MDR1* overexpressing cells generally displayed little or no staining. Intense staining was only rarely found in single cells. The values obtained by spectroscopy for the different samples are thus due to all cells in a sample, rather than to a subpopulation.

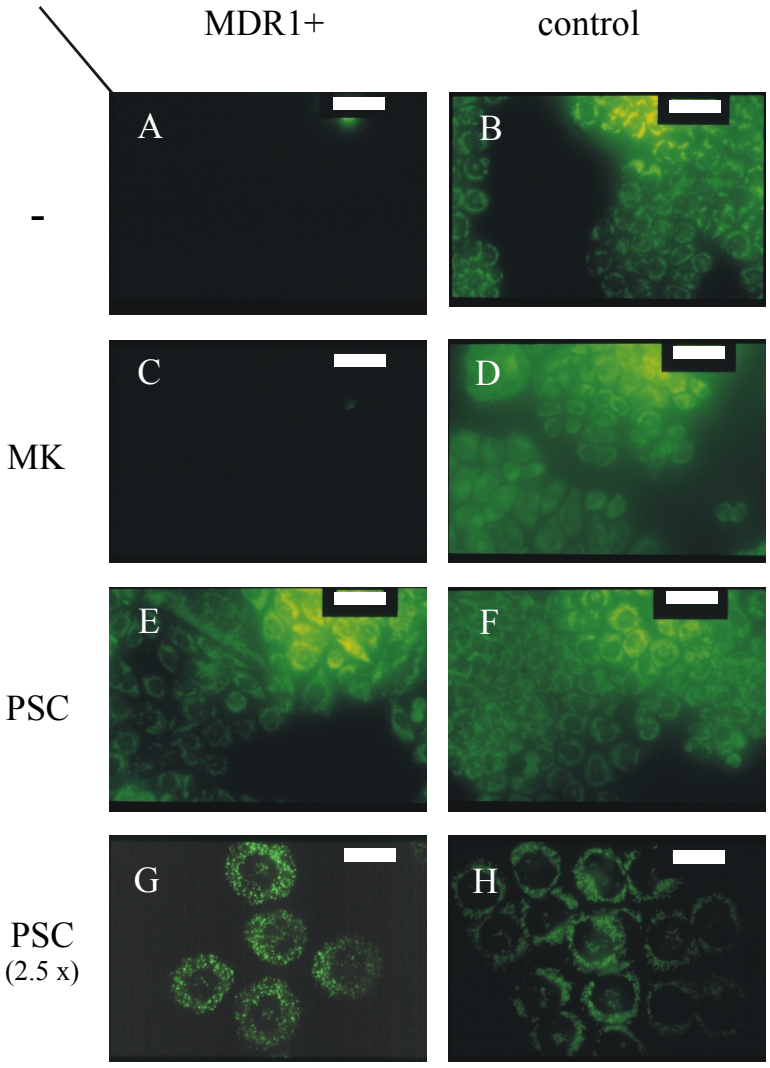

Fig.15: Accumulation of Rho 123 in *MDR1* overexpressing and control EPG85-257 cells at 37°C, fluorescence microscopy.

After inhibitor preincubations, *MDR1* overexpressing (A, C, E, G) and control (B, D, F, H) cells were labelled with Rho 123 for 60' in the absence (A, B) or presence of the MDR inhibitors MK 571 (C, D) and PSC 833 (E, F, G, H) as described in chapter 5. Cells were washed directly after labelling and green Rho 123 fluorescence was observed via fluorescence microscopy. Labelling concentrations and exposure times are identical for images A to F. Bar corresponds to 8 µm for images A-F and to 20 µm for images G and H.

Upon addition of the MDR1 Pgp inhibitor PSC 833, *MDR1* overexpressing cells showed similarly strong intracellular fluorescence as controls, whereas addition of the MRP1 inhibitor MK 571 had no visible effect on Rho 123 fluorescence. Fluorescence in controls was not affected by PSC 833 or MK 571. These microscopic findings support the results obtained by spectroscopy. The slight inhibitory effect of MK 571 on controls seen in spectroscopy was not observed here, probably a consequence of the qualitative rather than quantitative character of fluorescence microscopy.
Increased optical magnification allowed identification of the stained structures. In both untreated controls and in PSC 833 treated *MDR1* overexpressing cells and controls, Rho 123 selectively stained granular or serpentine particles arranged around the nucleus, and in a ring surrounding the Golgi apparatus. These structures have been identified as mitochondria using MitoTracker Red CMX-Ros (6.2). This corresponds with reports in literature describing Rho 123 as a mitochondrial marker (Ronot, 1986).

6.3.2 Outward transport of GS-MF

5-chloromethylfluorescein diacetate (CMFDA) is a non-fluorescent compound which crosses the plasma membrane and is cleaved by intracellular esterases to yield the fluorescent 5-chloromethylfluorescein (CMF) (Fig.16). CMF reacts with intracellular thiols and forms a fluorescent peptide-S-MF conjugate (e.g. Glutathione-methylfluoresceine (GS-MF), the formation of which is catalyzed by a glutathione S-transferase) (Roelofsen, 1997). As GS-MF does not easily cross the plasma membrane by itself, it is used as a long-term cell tracer (Molecular_Probes, 2001).

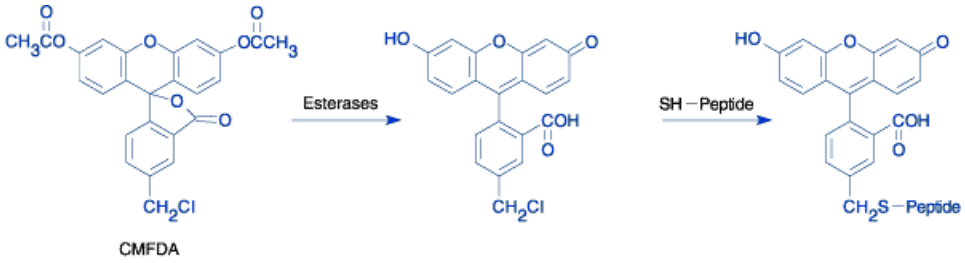

Fig.16: Structural formulae of CMFDA, CMF and a Peptide-S-MF (e.g. GS-MF).

CMF and GS-MF, but not CMFDA, are fluorescent. (taken from (Molecular_Probes, 2001)

MRP1 mediates outward transport of GS-MF and other glutathione-conjugates across the plasma membrane, which allows the use of GS-MF as a model MRP1 substrate (Roelofsen, 1997). Intracellular fluorescence after CMFDA loading was 6 a.u. for *MDR1* overexpressing and 8 a.u. for control cells. When the medium was removed immediately after washing, less than 5% of the accumulated GS-MF was found in the medium. During a 30 minute incubation at 37°C, both *MDR1* overexpressing and control cells showed comparable outward transport of GS-MF, over 85% of the accumulated GS-MF being expelled into the medium (Fig.17).

In the presence of the MRP1 inhibitor MK 571, GS-MF transport was pronouncedly reduced and comparable in both sublines, only 47% and 57% of GS-MF still reaching the medium in *MDR1* overexpressing and control cells, respectively. The MDR1 Pgp inhibitor PSC 833 decreased GS-MF outward transport in the *MDR1* overexpressing subline (56% in the medium), while only slightly decreasing transport in control cells (76% in the medium), which suggests low transport affinity of MDR1 Pgp for GS-MF.

Both cell lines possess similar MRP1 activity, which is specifically inhibited by MK 571, while PSC 833, a potent inhibitor of MDR1 Pgp, also reveals some efficiency to inhibit MRP1, confirming previous findings (Lehne, 2000).

In microscopy experiments, all probes exhibited similar, strong fluorescence directly after labelling. When cells were incubated further in the absence of CMFDA, controls showed strong cytosolic retainment of GS-MF (Fig.17), while fluorescence was somewhat reduced in the cytosol of *MDR1* overexpressing cells.

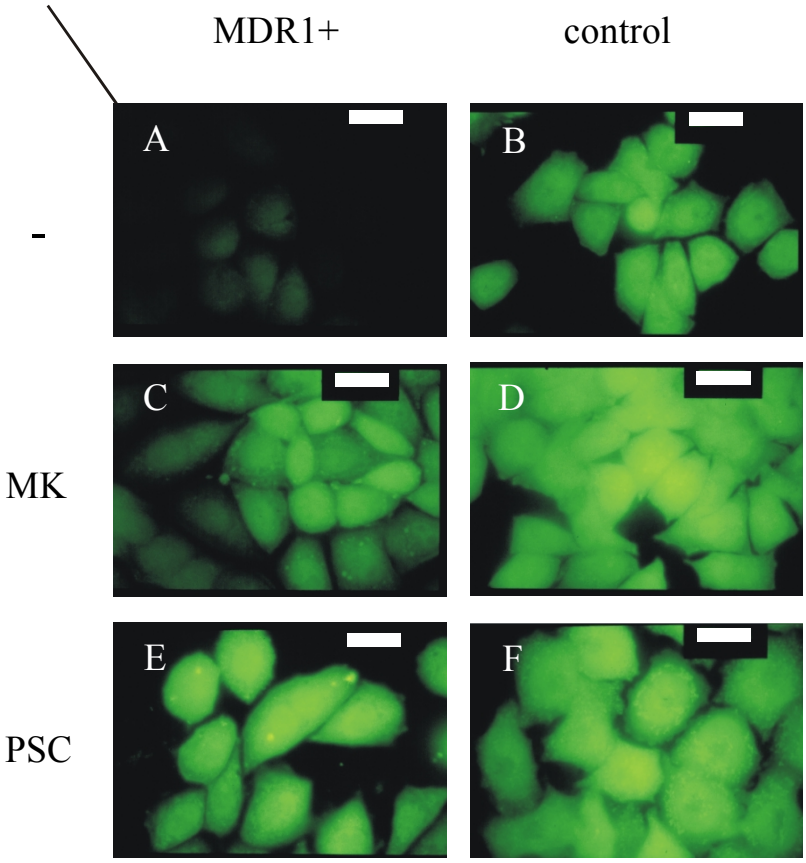

Fig.17: Outward transport of GS-MF in *MDR1* overexpressing and control EPG85-257 cells at 37°C, fluorescence microscopy.

After inhibitor preincubations, *MDR1* overexpressing (A, C, E) and control (B, D, F) cells were labelled with CMFDA for 15 minutes, washed, incubated for further 60 minutes in the absence (A, B) or presence of the MDR inhibitors MK 571 (C, D) and PSC 833 (E, F) as described in chapter 5. Prior to microscopic observation of green GS-MF fluoescence, cells were again washed. Labelling concentrations and exposure times are identical for all images shown. Bar, 20 µm.

When the MRP1 inhibitor MK 571 was present, *MDR1* overexpressing cells also displayed high cytosolic GS-MF fluorescence, controls retained equally high fluorescence as without inhibition. The MDR1 Pgp inhibitor PSC 833 increased GS-MF fluorescence in both cell lines. In contrast to Rho 123, GS-MF stains the whole cytosol, giving the cells a balloon-like appearance. In less intensely labelled probes (e.g. non-inhibited *MDR1* overexpressing cells), the nucleus and granular structures which might represent mitochondria appeared slightly more fluorescent than the rest of the cytosol. In a number of probes, the Golgi apparatus appeared to be free from fluorescence.

Both the *MDR1* overexpressing and control EPG 85-257 cells show GS-MF transporting MRP1 activity, which corresponds with similar synthesis of MRP1 in these cells (Pohl, 2002), 6.1. MRP1 activity was homogenous for a cell population. The MRP1 inhibitor MK 571 strongly inhibited this activity. The MDR1 Pgp inhibitor PSC 833 also significantly inhibited GS-MF outward transport, though to a lesser degree than MK 571.
Rather low, unspecific inhibition of MRP1 by PSC 833 has been described (Barrand, 1993). However, this does not appear to be of great importance here, as PSC 833 only marginally inhibits outward transport of GS-MF in controls.
More likely, MDR1 Pgp partially contributes to outward transport of GS-MF, since inhibition by PSC 833 is markedly higher in *MDR1* overexpressing cells than in controls. The similar retention levels of GS-MF in both uninhibited *MDR1* overexpressing and control cells do not rule out this possibility, as GS-MF might already be in a minimal concentration range where the presence of additional transporters would not further decrease GS-MF levels in a significant way.

6.4 Outward Transport of C6-NBD-PC and -PE and Accumulation of -DG

To examine outward transport of C6-NBD-PC and -PE by MDR1 Pgp in *MDR1* overexpressing cells and controls, cells were incubated with the precursor lipid C6-NBD-PA for 180 minutes at 15°C where vesicular traffic is blocked (van Genderen, 1995). C6-NBD-PA is partially converted to C6-NBD-diacylglycerol (-DG) which crosses the plasma membrane (chapter 2.4) and becomes available for intracellular synthesis of C6-NBD-PC and -PE on the cytoplasmic leaflet of the ER (Fig.18) (Pagano, 1981). Additionally, the accumulation of C6-NBD-DG was assessed.

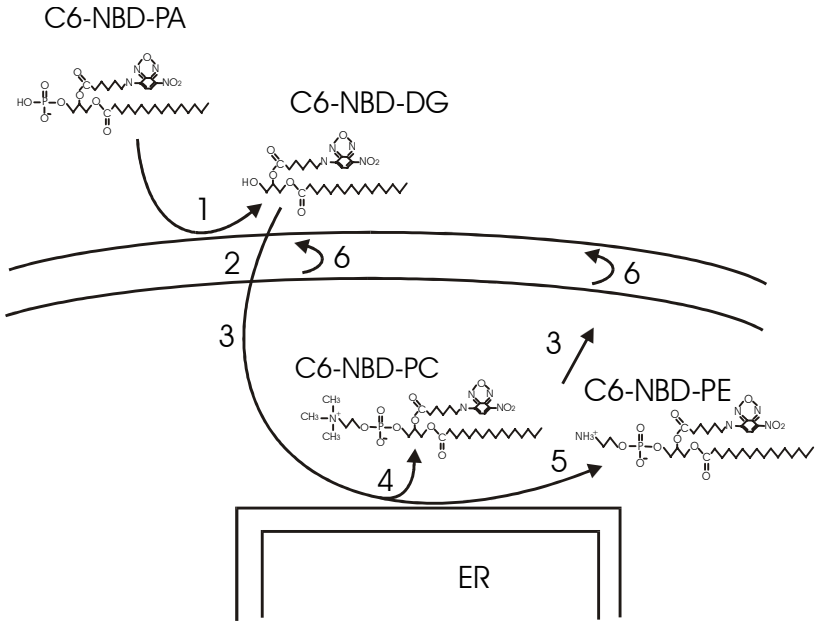

Fig.18: Schematic representation of C6-NBD-PC and -PE synthesis and outward transport.

On the plasma membrane, C6-NBD-PA is converted to -DG (dephosphorylation) by a phosphatidate phosphatase (1). Due to its small, uncharged headgroup, C6-NBD-DG can rapidly move across the plasma membrane (2) and, presumably by monomeric transport, distribute to different intracellular membranes (3). On the cytoplasmic leaflet of the ER, part of C6-NBD-DG is converted to -PC and -PE by phosphocholine transfer (4), respectively by phosphoethanolamine transfer (5) by the PC- and PE-synthases (Pagano, 1983). Synthesized C6-NBD-PC and -PE can again reach the inner leaflet of the plasma membrane by monomeric transport (3) and be transported to the outer leaflet by MDR1 Pgp (6).

6.4.1 Synthesis of C6-NBD-DG from -PA, and of C6-NBD -PC and -PE from -DG

During the incubation, about 16% of C6-NBD-PA was directly or indirectly converted to other lipid species (about 14% to -DG, 2% to -FA, less than 1% to -PC and -PE).
The total amount of C6-NBD-DG in cells and media was somewhat lower per dish (1.5 x 10^6 cells) for *MDR1* overexpressing cells than for controls (Fig.19). PSC 833 decreased the amount of C6-NBD-DG in cells and media only for *MDR1* overexpressing cells.
The total amount of C6-NBD-PC in *MDR1* overexpressing cells and media (23 pmol), was only about half of that in control cells and media (44 pmol).

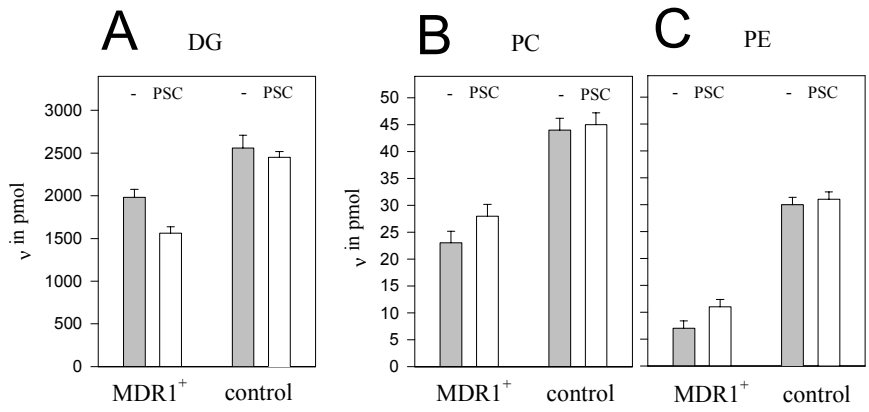

Fig.19: Synthesis of C6-NBD-DG, -PC and -PE from C6-NBD-PA in *MDR1* overexpressing cells and control EPG85-257 cells.

MDR1 overexpressing and control cells were preincubated without inhibitors or with PSC 833 or MK 571, followed by an incubation with C6-NBD-PA for 180 minutes at 15°C in the presence or absence of the respective inhibitor. To trap the fluorescent lipid products (C6-NBD-DG, C6-NBD-PC and C6-NBD-PE) appearing on the cell surface in the medium, incubation was performed in the presence of BSA. Lipids were quantified as described in chapter 5. Graph shows total amounts of synthesized C6-NBD-DG (A), C6-NBD-PC (B), C6-NBD-PE (C) per dish. Data represent mean ± range of n = 1 experiment in duplicate (A), and mean ± S.E.M of at least n = 3 independent experiments in duplicate (B, C).

PSC 833 increased this amount for *MDR1* overexpressing cells (28 pmol), but not for control cells (45 pmol). The amount of C6-NBD-PE found in cells and media for *MDR1* overexpressing cells (6 pmol) was only a fifth of the amount found for control cells (30 pmol). In the presence of PSC, this amount increased for *MDR1* overexpressing cells (11 pmol), but not for control cells (31 pmol).

6.4.2 Outward transport of C6-NBD-PC and -PE by MDR1 Pgp

C6-NBD-PC and -PE, synthesized on the cytoplasmic leaflet of the ER, were originally present exclusively in the cell. Nevertheless, as much as 64% of C6-NBD-PC and 79% of C6-NBD-PE were extracted into the BSA medium of the *MDR1* overexpressing subline, while the BSA medium of control cells contained only a small fraction of each lipid (17% of C6-NBD-PC and 6% of C6-NBD-PE) (Fig.20).

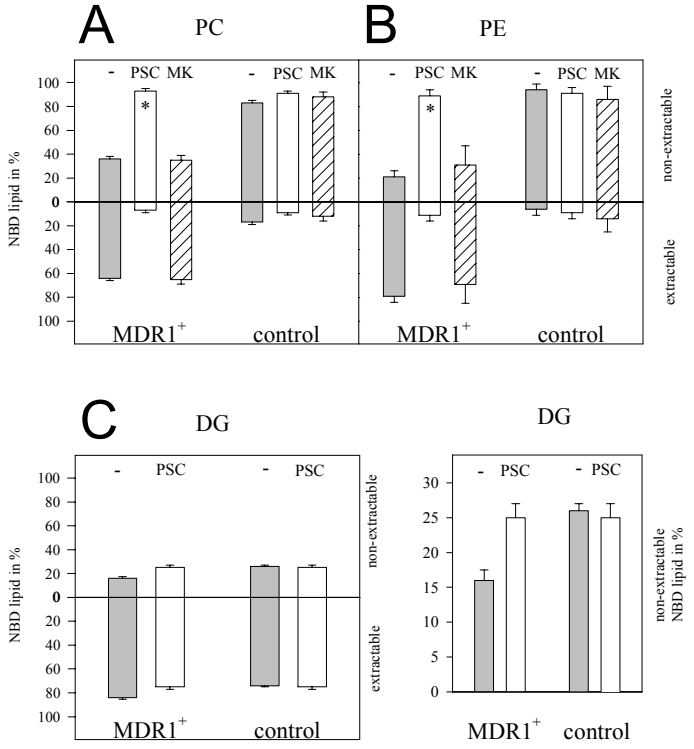

Fig.20: Outward transport of C6-NBD-PC and -PE and accumulation of C6-NBD-DG in *MDR1* overexpressing and control EPG85-257 cells.

Experimental procedures for outward transport of C6-NBD-PC (A), C6-NBD-PE (B), and accumulation of C6-NBD-DG (C) were as described in Fig.19.
A, B: Data represent mean ± S.E.M. of at least n = 3 independent experiments in duplicate; for MK 571 mean ± range of n = 1 experiment in duplicate. C: Data represent mean ± range of n = 1 experiment in duplicate. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) without inhibitor pretreatment ($p<0.05$).

Addition of PSC 833 caused a significant decrease in the amount of C6-NBD-PC and -PE extracted into the medium of *MDR1* overexpressing cells (7% of C6-NBD-PC and 11% of C6-NBD-PE), while the amount of the two lipids extracted into the BSA medium of control cells was not significantly altered (9% of C6-NBD-PC and 9% of C6-NBD-PE). In both cell lines, MK 571 did not significantly affect transport of C6-NBD-PC and -PE to the cell surface (65% of C6-NBD-PC and 69% of C6-NBD-PE in the medium for *MDR1* overexpressing cells, and 12% of C6-NBD-PC and 14% of C6-NBD-PE for controls).

6.4.3 Decreased accumulation of C6-NBD-DG by MDR1 Pgp

C6-NBD-DG, unlike C6-NBD-PC and -PE, is synthesized on the plasma membrane. After the incubation, the major part of C6-NBD-DG (over 74%) was found in the BSA medium. As C6-NBD-DG reaching the outer membrane leaflet due to outward transport could not be distinguished from analog having remained on the outer leaflet or in the BSA medium, differences in the accumulation of C6-NBD-DG could reflect outward transport of C6-NBD-DG as well as its own synthesis or metabolic conversion into other lipid species. After 180 minutes at 15°C, the percentage of C6-NBD-DG inside of the cells was lower for *MDR1* overexpressing cells (16%) than for control cells (26%) (Fig.20). PSC 833 increased this percentage for *MDR1* overexpressing cells (25%), but not for controls (25%), suggesting decreased conversion of C6-NBD-DG into other lipid species upon removal from the respective enzymes by outward transport.

6.5 Outward Transport of C6-NBD-SM, -GlcCer and Accumulation of -Cer

For investigation of C6-NBD-SM and -GlcCer outward transport in *MDR1* overexpressing cells and in controls, cells were labelled with the precursor lipid C6-NBD-Cer for 180 minutes at 15°C, where vesicular traffic is blocked (van Genderen, 1995). C6-NBD-Cer crosses the plasma membrane (chapter 2.4) and reaches intracellular membranes, e.g. the Golgi membrane, where part of it is converted into C6-NBD-SM (lumenal leaflet) and -GlcCer (cytoplasmic leaflet) (Fig.21). Additionally, the accumulation of C6-NBD-Cer was studied in *MDR1* overexpressing cells and in controls.

6.5.1 Synthesis of C6-NBD-SM and -GlcCer from C6-NBD-Cer

Less than 4% of the C6-NBD-Cer added was converted to other lipid species by cellular enzymes (Table 4). Metabolic conversion was far lower in *MDR1* overexpressing cells than in controls.

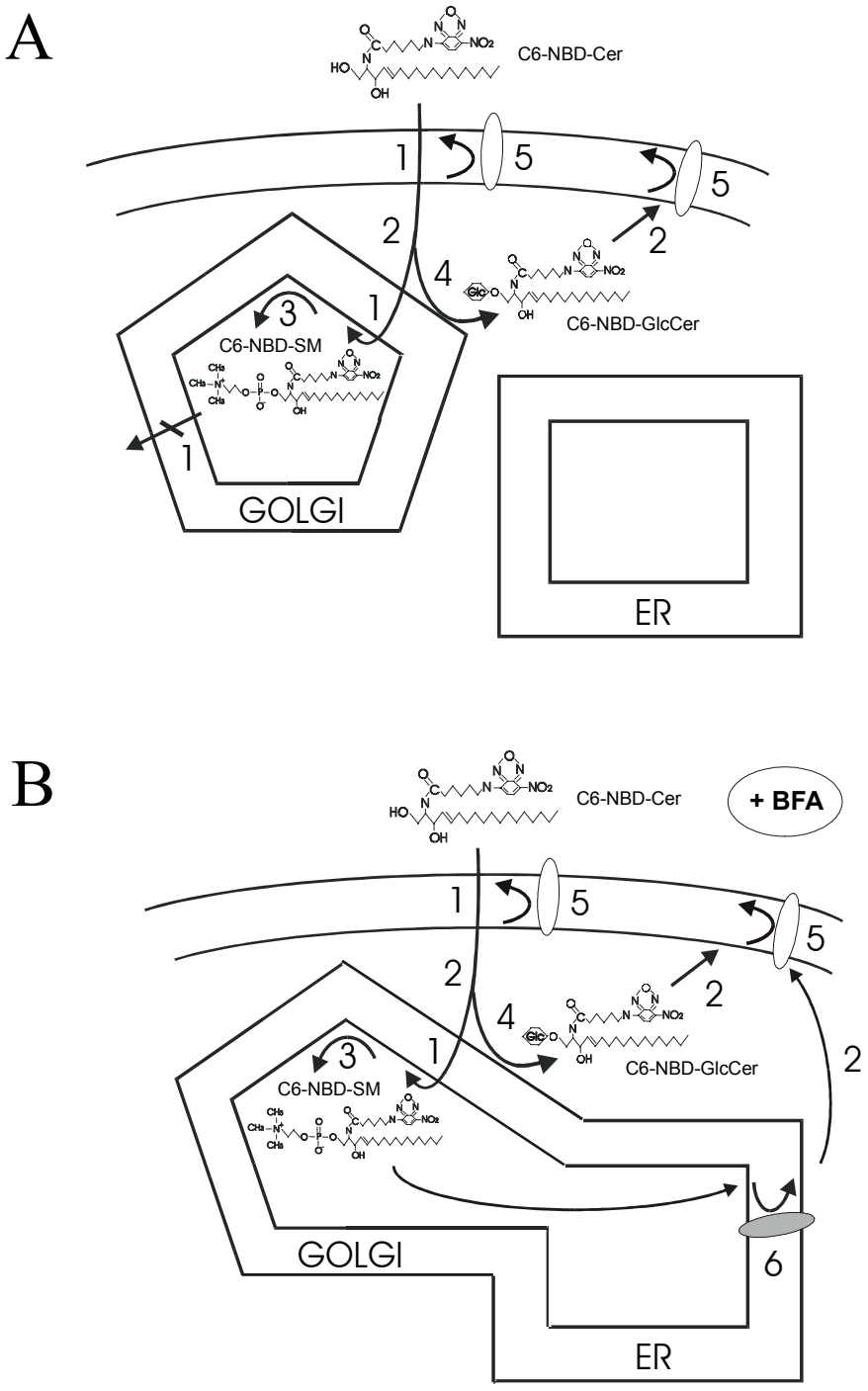

Fig.21: Schematic representation of C6-NBD-SM and -GlcCer synthesis and outward transport in the absence and presence of BFA.

Lipid synthesis and outward transport processes are shown in the absence (A) and presence (B) of BFA. C6-NBD-Cer rapidly crosses the plasma membrane (1) and distributes to different intracellular membranes, very likely by monomeric transport (2). After crossing the Golgi membrane, part of the C6-NBD-Cer is converted to C6-NBD-SM by transfer of phosphocholine derived from PC (3), which is catalyzed by the SM synthase (Sadeghlar, 2000). C6-NBD-SM does not easily traverse the Golgi membrane and remains trapped in the Golgi lumen at temperatures inhibiting vesicular transport (15°C) (A).

Some of the C6-NBD-Cer in the cell follows a second metabolic pathway: On the cytoplasmic leaflet of the Golgi, it is partially converted to C6-NBD-GlcCer (4) by the GlcCer synthase, transferring glucose from UDP-glucose to C6-NBD-Cer (Ichikawa, 1996). C6-NBD-GlcCer has again access to the inner leaflet of the plasma membrane by monomeric transport (2), where it is accessible to outward transport by plasma membrane transporters (5). The antibiotic Befeldin A (BFA) has been reported to affect transport vesicle formation and budding in the Golgi apparatus, thus stopping anterograde vesicular transport between the compartments of the Golgi. As a consequence, the Golgi redistributes into the ER (B), and loses its access to the TGN (Sadeghlar, 2000).
C6-NBD-lipid analogs formerly trapped in the Golgi can now cross the membrane of the newly formed organelle via an ER descending translocase of low lipid specificity (6) (Herrmann, 1990), (Lippincott-Schwartz, 1990), (Kok, 1992), (van Helvoort, 1997). From the cytoplasmic side of the ER-Golgi, they can reach the inner leaflet of the plasma membrane, presumably as monomers (2). At temperatures inhibiting vesicular transport (below 15°C (van Genderen, 1995)) but in the presence of BFA, newly synthesized C6-NBD-SM, like -GlcCer, can thus become accessible to outward transport by plasma membrane transporters (5). In addition to the major SM synthase found on the lumenal side of the Golgi, van Helvoort et al. have reported the activity of a plasma membrane located SM synthase (van Helvoort, 1994). This additional SM synthase could supply the plasma membrane with C6-NBD-SM which would be directly available for outward transport by MDR1 Pgp.

Table 4: Amounts of C6-NBD-SM, -GlcCer, and -Cer in *MDR1* overexpressing and control EPG85-257 cells.

	extractable (pmol)			non-extractable (pmol)		
	SM	**GlcCer**	**Cer**	**SM**	**GlcCer**	**Cer**
MDR1$^+$	0.5± 0	11.6±0.7	3775± 89	3.2	1.8± 0	171± 4
MDR1$^+$ + PSC 833	0.9±0.5	1.1±0.7	3303± 26	13.4±0.7	70.4±2.1	285± 2
MDR1$^+$ + BFA	11.8±0.5	15.3±0.2	3711±140	7.1±0.7	0.9±0.9	146±12
control	1.4±0.5	3.2±0.5	3259± 19	14.3±0.7	57.2±5.2	294±16
control + PSC 833	1.1±0.2	2.7±0.9	3169± 33	15.9	70.6±0.9	341± 2
control + BFA	3.6± 0	3.0±0.2	3539± 83	63.5±0.7	66.0±1.4	184± 7

Experimental procedures were as described in Fig.22. Data represent mean ± range of one representative experiment performed in duplicate, respectively single determinations where the range is not given.

The total amount of C6-NBD-SM in cells and media was 2 pmol for *MDR1* overexpressing cells and 26 pmol for controls (Fig.22). The antibiotic BFA, inducing redistribution of the Golgi into the ER (Sadeghlar, 2000), increased the amount of C6-NBD-SM in cells and media by several fold (17 pmol for *MDR1* overexpressing cells, 115 pmol for controls), which has been described to be a sign for BFA-induced morphological changes (Brüning, 1992). PSC 833 caused a several fold increase in the amount of C6-NBD-SM in cells and media for *MDR1* overexpressing cells (17 pmol), but not for controls (31 pmol).

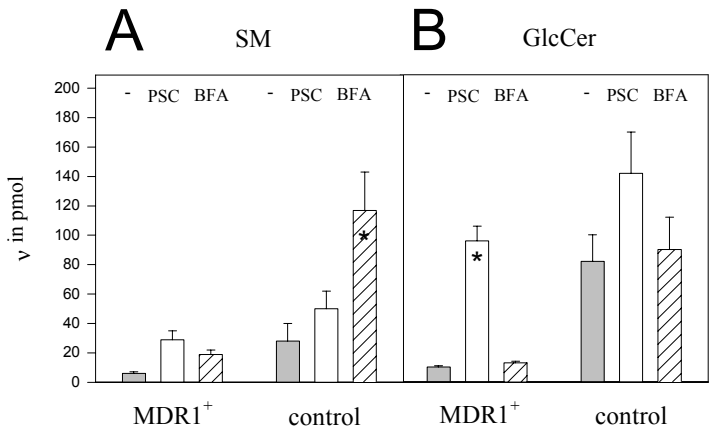

Fig.22: Synthesis of C6-NBD-SM and -GlcCer from C6-NBD-Cer in *MDR1* overexpressing cells and control EPG85-257 cells.

Cells were preincubated without additives or with PSC 833 or BFA, followed by an incubation with C6-NBD-Cer for 180 minutes at 15°C in the presence or absence of the respective additive. To trap the fluorescent lipid products (C6-NBD-SM and C6-NBD-GlcCer) appearing on the cell surface in the extracellular medium, incubation was performed in the presence of BSA. Lipids were quantified as described in chapter 5. Graph shows total amounts of synthesized C6-NBD-SM (A) and C6-NBD-GlcCer (B) per dish. Data represent mean ± S.E.M. of at least n = 3 independent experiments in duplicate; for PSC 833 mean ± S.E.M. of at least n = 2 experiments in duplicate. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) without PSC 833 and BFA ($p<0.05$) determined by a 2-way analysis of variance Tukey test (Jandel SigmaStat 2.0) considering treatment and day of experiment.

C6-NBD-GlcCer was the major metabolic product: In *MDR1* overexpressing cells, 9 pmol C6-NBD-GlcCer were found in cells and media, the amount in controls being significantly higher (76 pmol). BFA did not significantly affect this amount (11 pmol for *MDR1* overexpressing cells, 89 pmol for controls), corresponding with literature (Brüning, 1992). As the ER is a membrane system about 10 times the size of the Golgi apparatus (Brüning, 1992), the SM synthase which resides in the Golgi lumen could experience a several-fold increase in available substrate (C6-NBD-Cer) through the action of BFA, resulting in an increase in synthesis of C6-NBD-SM. Meanwhile, the GlcCer synthase resides on the cytoplasmic leaflet of the Golgi with access to monomeric C6-NBD-Cer from the cytosol even when no BFA is present.

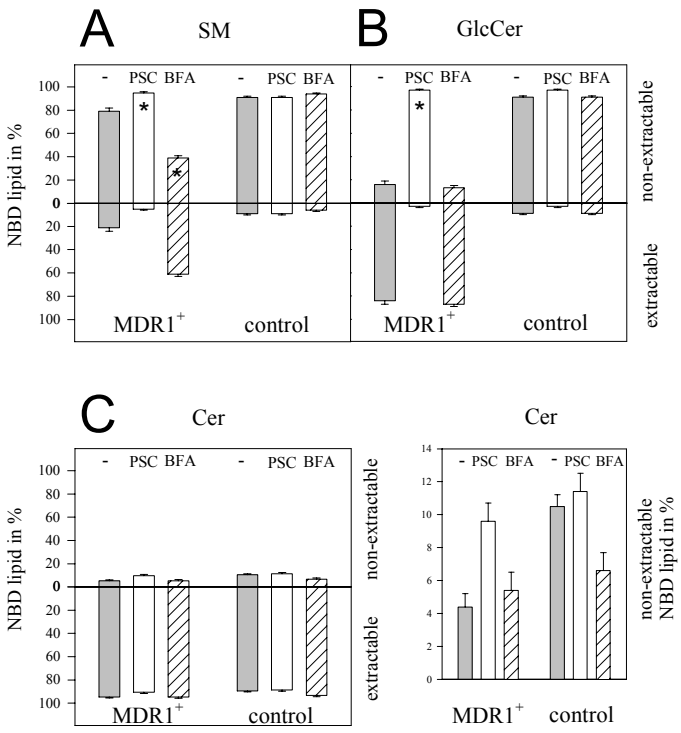

Fig.23: Outward transport of C6-NBD-SM and -GlcCer and accumulation of C6-NBD-Cer in *MDR1* overexpressing cells and control EPG85-257 cells.

Experimental procedures for outward transport of C6-NBD-SM (A), C6-NBD-GlcCer (B), and accumulation of C6-NBD-Cer (C) were as described in Fig.22. Data represent mean ± S.E.M. of at least n = 3 independent experiments in duplicate; for PSC mean ± S.E.M. of at least n = 2 experiment in duplicate, for Cer mean ± range of at least n = 2 experiments in duplicate. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) without PSC 833 or BFA ($p<0.05$).

PSC 833 increased the amount of C6-NBD-GlcCer in cells and media very strongly in *MDR1* overexpressing cells (78 pmol) and moderately in controls (124 pmol).

6.5.2 Outward transport of C6-NBD-SM and -GlcCer by MDR1 Pgp

Initially, C6-NBD-SM and -GlcCer were present entirely inside of the cell, their place of synthesis being the lumenal and the cytoplasmic leaflet of the Golgi, respectively. After 180 minutes, 21% of C6-NBD-SM were extracted into the BSA medium of *MDR1* overexpressing cells (Fig.23), while the BSA medium of control cells contained a significantly lower fraction (9%) of this lipid analog.

Part of the C6-NBD-SM extracted into the medium could originate from an additional SM synthase located in the plasma membrane (legend Fig.22), (van Helvoort, 1994).

The presence of PSC 833 significantly reduced outward transport of C6-NBD-SM in *MDR1* overexpressing cells (5% of C6-NBD-SM in the medium). In the medium of controls, the very low percentage of C6-NBD-SM was not decreased any further by PSC 833 (9% of C6-NBD-SM in the medium).

C6-NBD-SM became available for outward transport upon addition of BFA in the *MDR1* overexpressing cell line (61% in the BSA medium), but not in the control line (6% in the medium).

In *MDR1* overexpressing cells, as much as 84% of C6-NBD-GlcCer could be extracted by BSA during the incubation (Fig.23). In control cells, the percentage of extracted C6-NBD-GlcCer remained extremely low (9% in the medium).

The effect of PSC 833 on C6-NBD-GlcCer outward transport in *MDR1* overexpressing cells was dramatic: Here, PSC 833 suppressed the initially high outward transport of C6-NBD-GlcCer almost completely (3% of C6-NBD-GlcCer in the medium, significant difference versus *MDR1* overexpressing cells without inhibitor). In controls, however, PSC 833 had no significant influence on the already low outward transport of C6-NBD-GlcCer (3% of C6-NBD-GlcCer in the medium). BFA had no effect on the distribution of C6-NBD-GlcCer over the plasma membrane leaflets in either cell line (87% and 9% of C6-NBD-GlcCer in the medium of *MDR1* overexpressing cells and controls, respectively).

6.5.3 Decreased accumulation of C6-NBD-Cer by MDR1 Pgp

Following the incubation, the non-metabolized lipid analog C6-NBD-Cer used for labelling was still present mainly (over 88%) in the medium of both sublines (Fig.23). However, the fraction of C6-NBD-Cer inside of *MDR1* overexpressing cells was only about half of that found in controls (5.4% and 10.5% of C6-NBD-Cer remaining in *MDR1* overexpressing cells and controls, respectively).

In the presence of PSC 833, the fraction of C6-NBD-Cer in the cells was increased by about 2-fold in *MDR1* overexpressing cells (9.6% of C6-NBD-Cer in cells), but only slightly in controls (11.4% of C6-NBD-Cer in the cells).

Although PSC 833 has been claimed to increase Cer synthesis independent of MDR1 Pgp (Goulding, 2000), re-synthesis of C6-NBD-Cer from C6-NBD-SM and -GlcCer appears to be of minor importance in EPG 85-257 cells: The amount of C6-NBD-Cer accumulated in *MDR1* overexpressing cells increased in the presence of PSC 833, while the total amount of C6-NBD-SM and -GlcCer increased as well. In controls, no change in C6-NBD-Cer accumulation was seen in the presence of PSC 833.

Upon addition of BFA, the fraction of C6-NBD-Cer present in the cells decreased slightly in *MDR1* overexpressing cells, and somewhat more in control cells (5.4% and 6.6% of C6-NBD-Cer in *MDR1* overexpressing and control cells, respectively), possibly due to high conversion into C6-NBD-SM.

6.5.4 Intracellular localization of C6-NBD-Cer and its metabolic products

While a scattered region in proximity to the nucleus was labelled by C6-NBD-Cer and its metabolic products in *MDR1* overexpressing cells (Fig.24), the labelled region appeared more distinct in controls (Fig.24). Depending on the concentration used, weak fluorescence could also be observed in fine, mesh-like cytosolic structures.

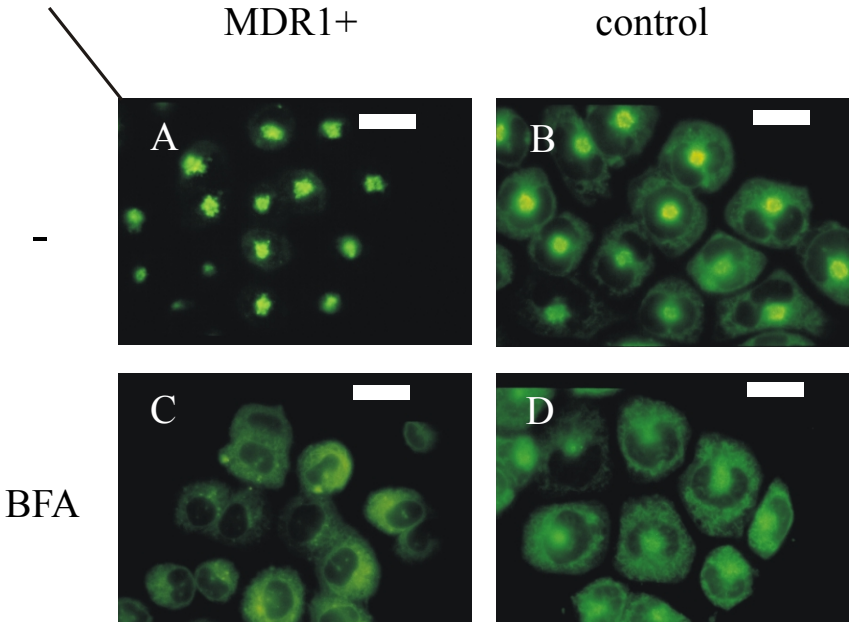

Fig.24: Labelling of *MDR1* overexpressing and control EPG85-257 cells with C6-NBD-Cer, fluorescence microscopy.

Following a preincubation without (A, B) or with BFA (C, D), cells were labelled for 10 minutes with C6-NBD-Cer in 0.1% BSA, incubated at 37°C without or with BFA for 60 minutes as described in chapter 5. For microscopic examination of *MDR1* overexpressing (A, C) and control (B, D) cells, cells were subjected to BSA back exchange and washed twice. Labelling concentrations and exposure times are identical for all images shown. Bar, 20 µm.

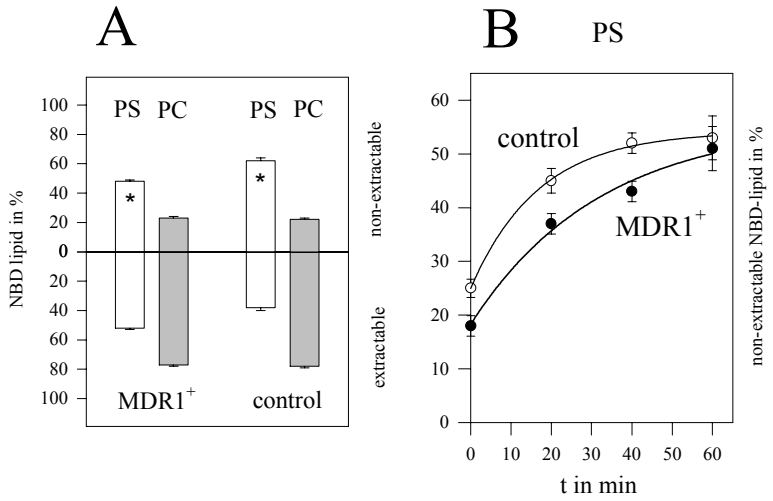

Fig.25: Inward transport of C6-NBD-PS and -PC in EPG85-257 cells.

MDR1 overexpressing and control cells were labelled with C6-NBD-PS or C6-NBD-PC, washed on ice, and BSA back exchange was performed either immediately (t = 0') or following incubation at 20°C for indicated lengths of time. (A) Inward transport of C6-NBD-PS and -PC after a 30 minute incubation. Lipids were quantified as described in chapter 5. For C6-NBD-PS, data represent mean ± S.E.M. of n = 3 independent experiments in duplicate. For C6-NBD-PC, results shown are mean ± S.E.M. of n = 2 independent experiments in duplicate. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) at t = 0' ($p<0.05$). (B) Time course of C6-NBD-PS inward transport. Lipids were extracted as described in chapter 5., dried, dissolved in chloroform/methanol (1:1) and quantified spectroscopically (no lipid separation). Data represent mean ± S.E.M. of at least n = 2 independent experiments in duplicate, for t = 60' mean ± range of n = 1 experiment in duplicate, and were fitted to a monoexponential function. Data shown in B are independent of data shown in A.

Similar structures as with C6-NBD-Cer were stained by the lectine TRX-WGA (6.2.2), confirming accumulation of C6-NBD-Cer and its metabolic products in the Golgi apparatus as described by Lipsky (Lipsky, 1985).
As labelling of the Golgi is based on different properties of the two markers, the differences in Golgi labelling between EPG 85-257 control cells and *MDR1* overexpressing cells probably reflect actual differences in the shape of the organelle, not previously reported for these two sublines.
When BFA was present, C6-NBD lipid fluorescence was still found in the region near the nucleus, however, the intensity appeared to be reduced. NBD fluorescence frequently increased in the mesh-like structures in both the cytosol of *MDR1* overexpressing cells (Fig.24) and controls (Fig.24) upon treatment with BFA. This pattern was similar to the labelling pattern seen in cells labelled with the ER stain FITC-ConA, supporting the redistribution of the Golgi (and the lipid analogs located in its lumen, in particular C6-NBD-SM) into the ER as reported by Sadeghlar (Sadeghlar, 2000).
Yet, this modification in the staining pattern was not always very distinct.

6.6 Inward Transport of C6-NBD-Lipid Analogs

Unlike for C6-NBD-PC and -PE, the lipid precursor C6-NBD-PA is not metabolically converted to the respective PS analog. In order to obtain labelling of the inner plasma membrane leaflet with C6-NBD-PS, we tested gastric carcinoma cells for aminophospholipid translocase activity. C6-NBD lipids were introduced into the exoplasmic leaflet, and probe remaining in the exoplasmic leaflet was removed at various times of incubation using BSA.

6.6.1 Inward transport of C6-NBD-PS, but not of -PC in gastric carcinoma cells

Indeed, we found rapid C6-NBD-PS inward transport in both cell lines, only 52% and 38% of the analog still being accessible to BSA extraction after 30 minutes at 20°C in *MDR1* overexpressing cells and control cells, respectively (Fig.25). PSC 833 did not affect C6-NBD-PS inward transport in either subline (not shown). The metabolic conversion of C6-NBD-PS following the 30 minute incubation at 37°C is shown in Table 5.

Table 5: Metabolic products of C6-NBD-PS in *MDR1* overexpressing and control EPG85-257 cells.

		% synthesized	
		t=0'	**t=30'**
MDR1+	**C6-NBD-PE**	6±1	24±1
	C6-NBD-PA	2±1	4±1
control	**C6-NBD-PE**	10±2	35±1
	C6-NBD-PA	4±1	3±1

Cells were labelled with C6-NBD-PS and incubated at 20°C for 30 minutes (t=0'), respectively incubated additionally at 37°C for 30 minutes (t=30'). Lipids were quantified as described in chapter 5. Formation of metabolic products other than C6-NBD-PE and -PA was negligeable. Results are shown as mean ± S.E.M. of at least n = 3 independent experiments performed in duplicate.

In contrast to the aminophospholipid analog C6-NBD-PS, the bulk (77-78%) of C6-NBD-PC was accessible to BSA in both cell lines even after 30 minutes at 20°C (versus 87 and 80% immediately after labelling in *MDR1* overexpressing cells and control cells, respectively).

6.6.2 Intracellular localization of C6-NBD-PS

Spectroscopy results were confirmed by microscopy: Upon incubation with C6-NBD-PS and -PC on ice, plasma membranes of *MDR1* overexpressing cells and controls became highly fluorescent. At 20°C, C6-NBD-PS rapidly appeared in the intracellular lumen (Fig.26). After a 30 minute incubation and BSA back exchange, fluorescence was observed in granular structures dispersed in the cytosol of both sublines, identified as mitochondria using MitoTracker Red CMX Ros (chapter 6.2.1, Fig.12).
Control cells additionally accumulated fluorescence in a globular region near the nucleus, identified as the Golgi by labelling with TRX-WGA (chapter 6.2.2, Fig.12) and C6-NBD-Cer (chapter 6.5.4, Fig.24), while this was rarely observed in *MDR1* overexpressing cells.

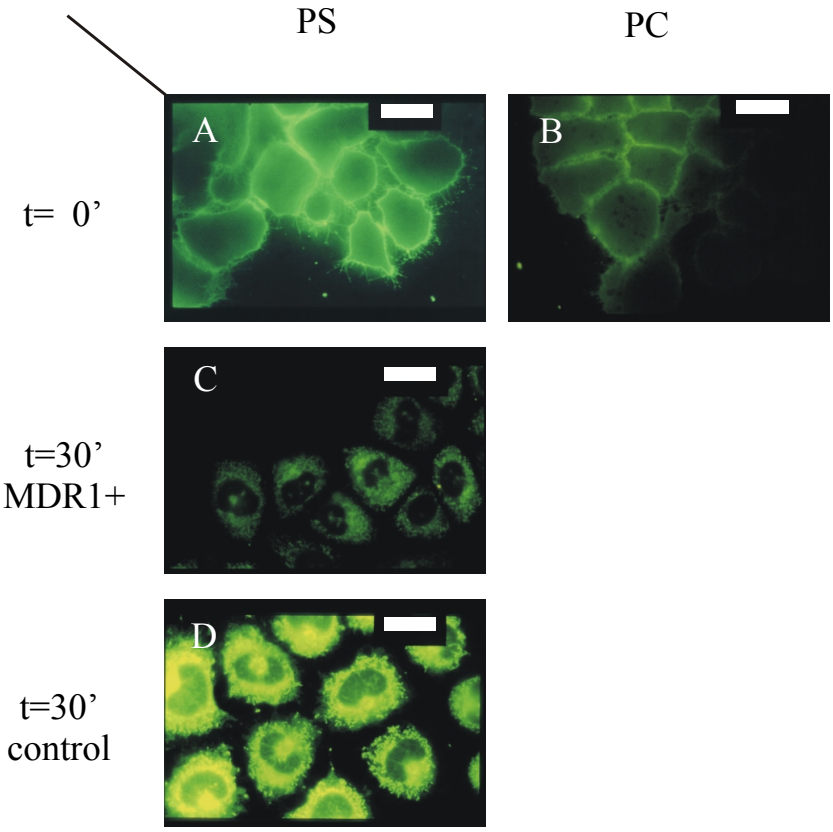

Fig.26: Labelling of EPG85-257 cells with C6-NBD-PS and -PC, fluorescence microscopy.

After labelling with 5 µM C6-NBD-PS or C6-NBD-PC for 20 minutes on ice, cells were washed, and immediately observed via fluorescence microscopy (controls: C6-NBD-PS (A), C6-NBD-PC (B)). Similar labelling as for controls was obtained in *MDR1* overexpressing EPG85-257 cells. Upon a 30 minute incubation at 20°C, BSA back exchange was performed, and *MDR1* overexpressing (C) and control cells (D) were observed via fluorescence microscopy. (C, D) were exposed twice as long as (A, B). Bar, 20 µm.

Yet, no colocalization of C6-NBD-PS was found with the ER marker FITC-ConA, apart from staining in the Golgi region produced by both compounds.
As outlined above, FITC-ConA presumably binds to smooth ER tubuli connected with the Golgi, but not to the Golgi itself, giving the staining a dispersed appearance. In contrast, C6-NBD-PS labelling of the region adjacent to the nucleus in controls appeared rather solid, confirming localization of the lipid analog in the Golgi itself. In summary, C6-NBD-PS does not stain all membrane systems present in the cell alike.

Table 6: Outward transport of C6-NBD-PS at 37°C in *MDR1* overexpressing and control EPG85-257 cells with and without inhibitors.

		NBD lipid % extractable
MDR1+	**-**	50±1 (16)
	MK	48±2 (4)
	PSC	28±2 (7)
	CsA	37±4 (2)
	Dex	39±4 (2)
	Gb	49±5 (1)
	BFA	64±3 (2)
control	**-**	29±1 (15)
	MK	22±2 (5)
	PSC	25±2 (6)
	CsA	24±4 (2)
	Dex	30±4 (2)
	Gb	23±5 (1)
	BFA	32±3 (2)

Measurement of C6-NBD-PS outward transport within a 30 minute incubation at 37°C was performed in the presence or absence of the MRP1 inhibitor MK 571, the ABCA1 inhibitor glyburide (Gb), the antibiotic Brefeldin A (BFA) or the MDR1 Pgp inhibitors PSC 833, cyclosporin A (CsA) and dexniguldipine-HCl (Dex). Lipids were extracted as described in chapter 5, dried, dissolved in chloroform/methanol (1:1) and quantified spectroscopically (no lipid separation). Results represent mean ± S.E.M. (Gb: mean ± range) of independent experiments performed in duplicate, the number of independent experiments is given in parentheses.

Instead, it is enriched in the mitochondria in both sublines, and in the Golgi in controls, while the ER remains essentially free from C6-NBD-PS. C6-NBD-PC, in contrast to C6-NBD-PS, remained confined to the plasma membrane of both control and *MDR1* overexpressing cells after incubation for 30 minutes at room temperature. After back exchange to BSA, only marginal cytoplasmic labelling was observed (not shown).

6.7 Outward Transport of C6-NBD-PS by MDR1 Pgp

Subsequent to intracellular labelling of cells with C6-NBD-PS, its transport back to the exoplasmic leaflet was determined. C6-NBD-PS and its metabolites appearing on the cell surface were trapped by BSA present in the incubation medium. After a 30 minute incubation at 37°C, lipid analogs were extracted from cells and medium, and analyzed by 2-D TLC (Table 6). In *MDR1* overexpressing cells, 53% of C6-NBD-PS was found in the medium, significantly more than in control cells (28%) (Fig.27).

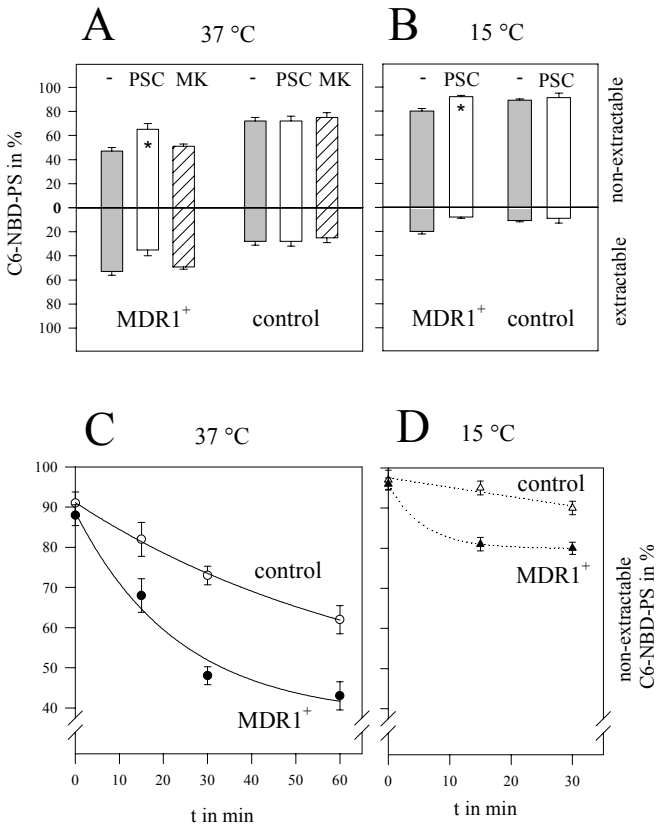

Fig.27: Outward transport of C6-NBD-PS in *MDR1* overexpressing and control EPG85-257 cells.

MDR1 overexpressing and control cells were preincubated without inhibitors or with PSC 833 or MK 571, labelled with C6-NBD-PS on ice for 15 minutes, and incubated at 20°C for 30 minutes to allow intracellular accumulation of the NBD analog. C6-NBD-PS remaining on the cell surface was then extracted twice by incubation with BSA in mPBS for 10 minutes on ice. Then, cells were incubated at 37°C (A, C) or 15°C (B, D) for indicated lengths of time (C, D) or for 30 minutes (A, B) in the presence of BSA to extract fluorescent lipids (C6-NBD-PS and metabolites) appearing on the cell surface into the medium. Lipids were analyzed as described in chapter 5. In panel A, data represent mean ± S.E.M. of at least n = 3 independent experiments in duplicate, for MK 571 mean ± S.E.M. of n = 2 experiments in duplicate. In panel B, results shown are mean ± S.E.M. of at least n = 2 independent experiments in duplicate, for PSC 833 mean ± range of n = 1 experiment in duplicate. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) without inhibitor pretreatment ($p<0.05$). In panels C and D, data are shown as mean ± S.E.M. of at least n = 2 independent experiments in duplicate, and fitted to a monoexponential function in C.

Even after 60 minutes, differences remained significant between *MDR1* overexpressing and control cells. Pretreatment with PSC 833 caused a sharp and highly significant decrease in the amount of C6-NBD-PS in the BSA medium of *MDR1* overexpressing cells (35%), while not affecting control cells.

Other MDR1 Pgp inhibitors (cyclosporin A, dexniguldipine-HCl) also blocked outward movement of C6-NBD-PS (not shown), while the MRP1 inhibitor MK 571, as well as glyburide, shown to inhibit ABCA1 (Marguet, et al., 1999), had no effect (Table 6). Expression of *ABCA1* is similarly weak in both sublines as indicated by RT-PCR analysis (D. Kerbiriou-Nabias and I. Laude, Fig.10). Increase of vesicular transport has been shown to be one mechanism by which some cells increase their drug tolerance (Dietel, 1990).

To investigate whether C6-NBD-PS transport observed at 37°C could be due to increased membrane traffic in the *MDR1* overexpressing subline, experiments were performed at 15°C. Although outward transport of the PS analog was reduced at this temperature, a clear difference in transport between control and *MDR1* overexpressing cells was observed: 20% and 11% of C6-NBD-PS were found in the medium of the *MDR1* overexpressing subline and the control cell line, respectively (Fig.27).

PSC 833 reduced the fraction of C6-NBD-PS in the medium of *MDR1* overexpressing cells to the level of controls, while no change in the distribution of C6-NBD-PS was observed when control cells were treated with PSC 833.

To ensure that C6-NBD-PS was accessible to outward redistribution, rather than confined to the Golgi compartment in particular in control cells, BFA was employed. When preincubated with BFA at 37°C for 30 minutes and when BFA was present during the outward transport assay, C6-NBD-PS outward transport was not affected significantly in control cells, while being slightly increased in *MDR1* overexpressing cells (Table 6). Fluorescence microscopy confirmed efficient outward transport of C6-NBD-PS in *MDR1* overexpressing cells in comparison to control cells.

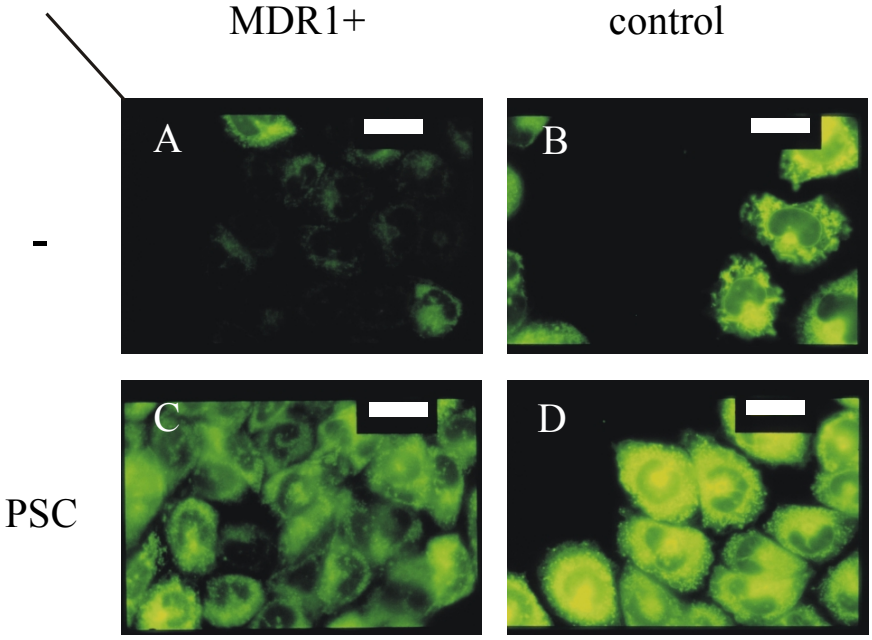

Fig.28: Outward transport of C6-NBD-PS in *MDR1* overexpressing and control EPG85-257 cells, fluorescence microscopy.

After labelling of the plasma membrane with C6-NBD-PS, the analog was allowed to accumulate intracellularly. Subsequently, incubation for outward transport was performed without inhibitors (A and B) or in the presence of PSC 833 (C and D) at 37°C as described in chapter 5. For microscopic examination of *MDR1* overexpressing (A, C) and control (B, D) cells, BSA was removed and cells were washed twice. Labelling concentrations and exposure times are identical for all images shown. Bar, 20 µm.

After labelling of the exoplasmic leaflet and subsequent inward transport of the analog at 20°C, *MDR1* overexpressing and control cells showed bright intracellular fluorescence. Subsequently, cells were incubated for 1 hour at 37°C in the presence of BSA and washed prior to examination by fluorescence microscopy.

MDR1 overexpressing cells lost most of the analog into the medium. while intracellular fluorescence decreased only slightly in control cells (Fig.28). In the presence of PSC 833, strong intracellular fluorescence was maintained in both *MDR1* overexpressing cells and controls. Meanwhile, the intracellular distribution of C6-NBD-PS to cell organelles did not change in either of the two sublines in response to PSC 833. Lipid metabolism was not responsible for the observed differences between control and *MDR1* overexpressing cells (Table 5).

6.8 Increased Exposure of Endogenous PS on *MDR1* Overexpressing Cells

Exposure of endogenous PS in *MDR1* overexpressing cells and controls was tested by flow cytometry. PS present on the outer plasma membrane leaflet of cells with intact cell membranes was detected by labelling with both FITC-Annexin V, a high affinity PS binding protein (Dachary-Prigent, 1993) and the membrane-impermeable nucleic acid stain propidium iodide. Regions were set to exclude subcellular particles, and only single cells were counted (10 000 cells / sample). A further gate excluded damaged cells with elevated propidium iodide staining.

Few ungated cells with propidium iodide permeable membranes were detected, slightly more in *MDR1* overexpressing cells (5%) than in controls (1%). Cells from the *MDR1* overexpressing subline bound over 2-fold more FITC-Annexin V than control cells. The mean FITC-Annexin V fluorescence intensity was (110±4) a.u. for *MDR1* overexpressing cells, and (46±5) a.u. for control cells (n = 10 000) (Fig.29), the differences being highly significant.

The FITC-Annexin V histogram shape was similar for both cell lines, showing that the amount of PS present on the outer plasma membrane leaflet in *MDR1* overexpressing cells was generally increased for all cells in this population. To assess whether FITC-Annexin V binding was reduced upon inhibition of MDR1 Pgp, cells were preincubated with PSC 833 for 30 minutes at 37°C. The mean FITC-Annexin V fluorescence intensity was significantly decreased in *MDR1* overexpressing cells (90±5) a.u., and remained unchanged in controls (46±6) a.u. However, FITC-Annexin V binding to PSC 833 treated *MDR1* overexpressing cells remained significantly higher in comparison to controls.

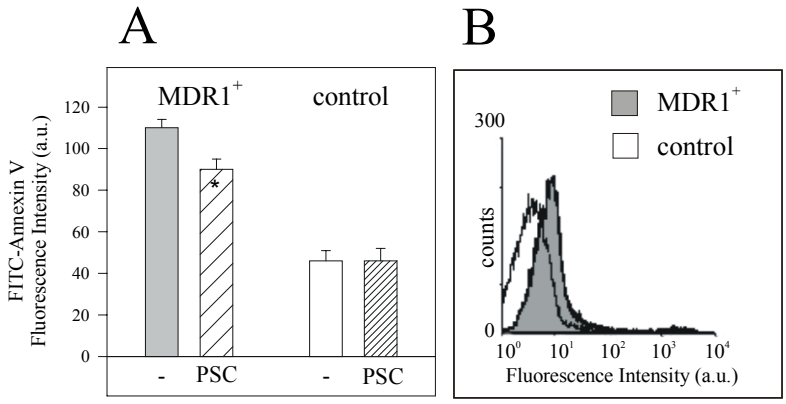

Fig.29: Exposure of endogenous PS on *MDR1* overexpressing and control EPG85-257 cells.

Flow cytometric analysis of FITC-Annexin V binding to the cell surface of *MDR1* overexpressing and control EPG85-257 cells. Following a 30 minute incubation with or without PSC 833 at 37°C, cells were colabelled with FITC-Annexin V and the membrane impermeable nucleic acid stain propidium iodide as described in chapter 5. Cells showing elevated propidium iodide staining were excluded. 10 000 cells were counted per sample. In the bar plot (A), mean ± S.E.M. of n = 7 independent experiments are represented, in the histogram (B), FITC-Annexin V binding to *MDR1* overexpressing cells (gray) and control cells (white) is shown in one typical experiment. The asterisks denote a significant difference compared to the respective cells (control or *MDR1* overexpressing cells) without inhibitor pretreatment ($p<0.05$).

6.9 Discussion

MDR1 Pgp has been implicated with the transport of lipids from the inner to the outer leaflet of the plasma membrane. In this chapter, the transport of lipids to the cell surface was investigated in *MDR1* overexpressing EPG85-257 human gastric carcinoma cells compared to controls.

In agreement with elevated synthesis of MDR1 Pgp in *MDR1* overexpressing EPG85-257 cells (Stein, 2002), increased outward transport of the MDR1 Pgp model substrate Rho 123 indicated high MDR1 Pgp activity in *MDR1* overexpressing EPG85-257 cells compared to the EPG85-257 control cell line.

Corresponding with only slightly elevated synthesis of the ABC transporter MRP1 in *MDR1* overexpressing cells versus controls (Stein, 2002), the activity of MRP1, potentially involved in lipid transport as well, was found to be similar for both sublines.
Expression of the gene coding for the ABC transporter ABCA1 was neglectable in both sublines (D. Kerbiriou-Nabias and I. Laude, personal communication).
These features make the human gastric carcinoma cell line EPG85-257P and its *MDR1* Pgp overepressing subline EPG85-257RDB a suitable system for studying MDR1 Pgp mediated transport of lipids.
Incubation of the cells with C6-NBD-PA at 15°C permitted investigation of the transport of C6-NBD-PC and -PE (synthesized from C6-NBD-PA via the intermediate -DG) from the cytoplasmic to the exoplasmic plasma membrane leaflet. Compared to controls, very strongly increased outward transport of C6-NBD-PC and -PE was found in *MDR1* overexpressing cells, surpassing previous findings in *MDR1* transfected pig kidney epithelial cells (van Helvoort, 1996) and supporting a role for MDR1 Pgp in transport of C6-NBD-PC and -PE to the cell surface. Consistent with this, outward transport of C6-NBD-PC and -PE was sensitive to MDR1 Pgp inhibitors, while an inhibitor of MRP1, effectively blocking the outward transport of the MRP1 substrate GS-MF, had no effect on the appearance of either NBD lipid on the cell surface.
Compared to controls, lower amounts of C6-NBD-PC and -PE were formed from C6-NBD-DG in *MDR1* overexpressing cells, in which synthesis of C6-NBD-PC and -PE could be partially restored by an MDR1 Pgp inhibitor.
In addition, accumulation of C6-NBD-DG was reduced in *MDR1* overexpressing cells versus controls, and was completely restored upon inhibition of MDR1 Pgp. These results suggest transport of C6-NBD-DG from the inner to the outer leaflet of the plasma membrane by MDR1 Pgp, which has not been reported previously. As a consequence of C6-NBD-DG outward transport, the amount of available substrate for the PC- and the PE-synthase would decrease.
Outward transport of C6-NBD-SM and -GlcCer could be studied upon incubation of *MDR1* overexpressing and control cells with their precursor C6-NBD-Cer at 15°C. In *MDR1* overexpressing cells, outward transport of C6-NBD-GlcCer was strongly increased compared to controls.

Similarly, when the antibiotic BFA induced redistribution of the Golgi into the ER, outward transport of C6-NBD-SM was far higher in *MDR1* overexpressing cells than in controls.

An inhibitor of MDR1 Pgp decreased outward transport of both C6-NBD-SM and -GlcCer in *MDR1* overexpressing cells, confirming C6-NBD-SM and -GlcCer outward transport by MDR1 Pgp as reported before (van Helvoort, 1996).

In *MDR1* overexpressing cells, synthesis of C6-NBD-SM and -GlcCer and accumulation of C6-NBD-Cer were lower than in controls, and could be restored by an inhibitor of MDR1 Pgp, strongly pointing to outward transport of C6-NBD-Cer by MDR1 Pgp. In the literature, this has not been reported so far, and indirect assays had previously not suggested transport of Cer by MDR1 Pgp (Sietsma, 2001).

In human breast adenocarcinoma cells, PSC 833 was described to increase Cer synthesis independent of MDR1 Pgp (Goulding, 2000).

Meanwhile, in EPG 85-257 cells, re-synthesis of C6-NBD-Cer from C6-NBD-SM and -GlcCer appears to be of minor importance: The amount of C6-NBD-Cer which accumulated in *MDR1* overexpressing cells increases in the presence of PSC 833, as well as the total amount of C6-NBD-SM and -GlcCer. In controls, no change in C6-NBD-Cer accumulation is seen in the presence of PSC 833.

Unlike in experiments regarding outward transport of C6-NBD-PC and -PE (see above and (van Helvoort, 1996)), C6-NBD-PA is not a suitable precursor to label the cytoplasmic leaflet with C6-NBD-PS.

Therefore, we aimed to insert C6-NBD-PS into the cytoplasmic leaflet of the plasma membrane via the aminophospholipid translocase activity, an ubiquitous fast mechanism transporting PS from the outer to the inner plasma membrane leaflet. Indeed, inward movement measurements at 20°C revealed fast internalization of C6-NBD-PS, while C6-NBD-PC remained mostly confined to the exoplasmic leaflet of the plasma membrane. Preferential internalization of the PS analog by transbilayer movement was confirmed by fluorescence microscopy and suggests the presence of an aminophospholipid translocase activity. This inward directed transport activity mediated rapid and extensive labelling of the inner plasma membrane leaflet with C6-NBD-PS, necessary for analyses of C6-NBD-PS outward transport.

Continuous incubation of cells in the presence of BSA permits monitoring of the outward movement of C6-NBD lipids without needing to take into account simultaneous inward movement. BSA acts as an extracellular sink by rapidly extracting C6-NBD lipids (Marx, 2000), excluding analogs from inward transport via the aminophospholipid translocase. Within 30 minutes at 37°, a significantly higher percentage of C6-NBD-PS became accessible to BSA in *MDR1* overexpressing cells compared to controls. This was confirmed by fluorescence microscopy. When vesicular transport was inhibited at 15°C (van Genderen, 1995), *MDR1* overexpressing cells still exhibited significantly higher transport of C6-NBD-PS to the cell surface than did control cells.

Redistribution of the Golgi into the ER induced by BFA did not influence outward transport of C6-NBD-PS in control cells, while it slightly increased outward transport in *MDR1* overexpressing cells. We could therefore exclude that variations in the intracellular localization of C6-NBD-PS between both sublines gave rise to differences in C6-NBD-PS outward movement. This is substantial since Golgi morphology appears to differ in controls and *MDR1* overexpressing cells.

Outward directed transport of C6-NBD-PS was efficiently decreased by MDR1 Pgp inhibitors, marking the involvement of MDR1 Pgp.

Recently, two other ABC transporters, MRP1 (ABCC1) and ABCA1, have emerged as further candidate lipid translocases. In studies with *MRP1* knockout mice, MRP1 mediated transport of C6-NBD-PC and -PS was reported in erythrocytes, but no changes in the distribution of endogenous PS were detected (Dekkers, 1998), (Kamp, 1998).

Raggers et al. (Raggers, 1999) observed transport of lipid analogs in *MRP1* transfected epithelial cells for sphingolipids with an NBD moiety. Using the MRP1 specific inhibitor MK 571, our studies clearly demonstrate that this ABC transporter is not involved in transport of C6-NBD-PS to the cell surface of *MDR1* overexpressing EPG85-257 cells.

ABCA1-dependent transport of PS has recently been shown for various mammalian cells (Marguet, et al., 1999), (Hamon, 2000). However, we have no indication for any involvement of ABCA1 in the enhanced transport of C6-NBD-PS in *MDR1* overexpressing EPG85-257 cells.

In both sublines used, ABCA1 mRNA is barely detectable (D. Kerbiriou-Nabias and I. Laude, personal communication) and C6-NBD-PS transport was not affected by glyburide, reported to inhibit ABCA1-dependent exposure of PS (Marguet, et al., 1999).

Taken together, our results show MDR1 Pgp to mediate outward directed transport of C6-NBD-PS. In a previous study, Bosch et al. (Bosch, 1997) could not detect transport of the long-chain analog C12-NBD-PS by MDR1 Pgp, assessing the accumulation of C12-NBD-phospholipid analogs in *MDR1* overexpressing and control cells. Unlike in our study, the aminophospholipid translocase activity could not be eliminated in the experimental setup of Bosch et al. While we cannot exclude that C12-NBD-PS might not be a substrate for MDR1 Pgp, C12-NBD analogs of PE and PC were shown to be transported by MDR1 Pgp (Bosch, 1997). This points out that a longer fatty acid chain in the sn2 position does not prevent recognition of the analogs by MDR1 Pgp. Since the affinity of the aminophospholipid translocase is about ten times lower for analogs of PE than for PS (Zachowski, 1986), it is possible that MDR1 Pgp mediated transport of C12-NBD-PS, but not -PE, is masked by an aminophospholipid translocase activity. C12-NBD-PS is unfortunately not suitable for the setup to measure outward movement of PS analogs used here, as extraction of this analog by BSA is less efficient than of C6-NBD-PS (Wüstner, 1998).

A different approach to selectively investigate the activity of MDR1 Pgp, excluding other potential lipid transporters, is to reconstitute MDR1 Pgp in liposomes. In a recent paper, Romsicki and Sharom (Romsicki, 2001) have studied transport of a number of short-chain and long-chain NBD analogs of PC, PE, PS, and SM in proteoliposomes reconstituted with MDR1 Pgp. The reconstituted MDR1 Pgp was predominantly oriented inside-out into the liposome membranes.

Presumably due to steric reasons, the transmembrane distribution of nearly all NBD lipids was shifted to the outer leaflet in the presence of MDR1 Pgp, prohibiting a direct comparison with protein-free liposomes. Nevertheless, when ATP was added, the portion of lipid analogs in the inner leaflet of MDR1 Pgp containing liposomes increased.

While this is principally in line with our observation, the increase in the inner leaflet by a few percent (maximum 5%) (Romsicki, 2001) was far less in comparison to the transport activity found for the *MDR1* expressing cell line.

This may raise concerns with respect to the preservation of MDR1 Pgp function in proteoliposomes as indicated by a recent study of Rothnie et al. (Rothnie, 2001). In this report, the transport of short-chain NBD analogs of PC, PE, and Cer was studied in MDR1 Pgp reconstituted liposomes.

A low percentage of the analogs (up to about 6%) also appeared to reorient across the membrane in the presence of MDR1 Pgp, in a process that was surprisingly found to be ATP independent. However, the authors note that the proteoliposome assay used may be subject to important technical limitations (e.g. inhibition of further lipid transport by increased lateral pressure in the inner membrane leaflet following initial transport). Nevertheless, such studies can give insight into the biophysical restrictions for MDR1 Pgp mediated transport: As found by Rothnie et al., physiological concentrations of cholesterol seem to be required to partially compensate for membrane perturbations by MDR1 Pgp. This interplay of MDR1 Pgp with specific lipid compounds might be a prerequisite for the transport of amphiphilic substrates. Moreover, an effect of lateral pressure in the membrane on MDR1 Pgp mediated lipid transport could either act on MDR1 Pgp directly (mechanosensitivity) or on the substrate lipids' propensity to redistribute towards the layer exhibiting lower packing. Similarly, outward transport of various endogenous lipids in cells might produce lipid crowding in the outer leaflet of the plasma membrane which would need to be compensated for.

Having established that MDR1 Pgp can transport C6-NBD-PS to the outer plasma membrane leaflet, we studied the exposure of endogenous PS on the cell surface. Consistent with a role of MDR1 Pgp in the transport of endogenous PS, binding of FITC-Annexin V to the cell surface of *MDR1* overexpressing cells was significantly higher than binding to control cells, and inhibition of MDR1 Pgp decreased FITC-Annexin V binding in *MDR1* overexpressing cells but not at all in controls.

MDR1 Pgp mediated transport of endogenous PS might provide an explanation for increased PS exposure on tumorigenic keratinocytes reported previously (Utsugi, 1991). However, our approach does not allow quantification of endogenous PS in the exoplasmic leaflet. The FITC-Annexin V binding assay has been reported to be sensitive to as little as 5 mol% PS (Stuart, 1995), and the disturbance of PS asymmetry here is most likely only partial.

7 Lipid Transport via BCRP in EPG85-257 Human Gastric Carcinoma Cells*

Transport of lipid analogs and lipids has been demonstrated for additional members of the ABC protein superfamily besides MDR1 Pgp (chapter 6), e.g. for MDR2/3 Pgp (Smith, 1994), (van Helvoort, 1996), MRP1 (Kamp, 1998), (Dekkers, 1998), and ABCA1 (Marguet, et al., 1999), (Hamon, 2000).

These so-called *full-size* transporters each contain two nucleotide binding domains and at least two transmembrane domains ($(TM\text{-}ABC)_2$, MRP1: $TM_0(TM\text{-}ABC)_2$, (Fig.6, chapter 3.1). However, *half-size* ABC transporters, containing only one nucleotide binding domain and one transmembrane domain (TM-ABC or ABC-TM, Fig.6, chapter 3.1), are possibly implicated in the transport of lipids as well. The half-size transporter White (ABCG1), for example, appears to play a role in the regulation of cholesterol and phospholipid transport in macrophages and possibly in other cells as well, as it is ubiquitously found in human tissues (Klucken, 2000).

This chapter focuses on the potential involvement of the recently discovered half-size ABC protein Breast Cancer Resistance Protein (BCRP, MXR, ABCG2) in lipid transport. Outward transport of the lipid analog C6-NBD-PS, and exposure of endogenous PS on the outer leaflet of the plasma membrane is studied in the *BCRP* overexpressing human gastric carcinoma subline EPG85-257RN.

BCRP, a member of the ABCG family, was first reported to be a transporter of xenobiotics and cytostatic drugs. Recently, accumulation of a fluorescent ceramide analog has been found to be reduced in a *BCRP* overexpressing cell line (Litman, 2000), which could point to active outward transport of this lipid analog by BCRP.

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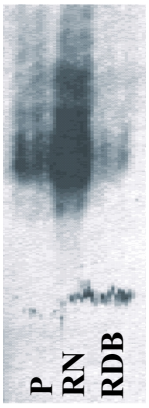
* Data in this chapter are part of a manuscript in preparation

Woehlecke, H., Pohl, A., Lage H., Herrmann, A. (2002): BCRP promotes transport of phospholipids in a human gastric carcinoma cell line, in preparation.

EPG85-257



BCRP

Fig.30: Northern blot hybridization of *BCRP* mRNA in EPG85-257 human gastric carcinoma cells.

Blots were probed with a 795 bp fragment of *BCRP* complementary DNA labelled with [^{32}P]deoxycytidine triphosphate. EPG85-257P (control), EPG85-257RN (*BCRP* overexpressing), EPG85-257RDB (*MDR1* overexpressing) (taken from: (Ross, 1999)).

7.1 The *BCRP* Overexpressing Human Gastric Carcinoma Cell Line EPG85-257RN

In vitro selection of EPG85-257P human gastric carcinoma cells with mitoxantrone, an anthraquinone derivative primarily used in the chemotherapy of breast cancer, yielded the *BCRP* overexpressing (Fig.30) (Ross, 1999) subline EPG85-257RN (Dietel, 1990). Synthesis of BCRP is 1.5 fold higher in *BCRP* overexpressing cells than in controls (Stein, 2002). *BCRP* overexpressing EPG85-257 cells tolerate a nearly 460-fold higher concentration of mitoxantrone than controls (Lage, 2000), and show increased resistance towards the anthracyclines doxorubicin and daunorubicin, but not towards vincristine and cisplatin. However, synthesis of MDR1 Pgp and MRP1 in these cells is also somewhat elevated versus controls (chapter 6.1, Fig.10) (Stein, 2002).

7.2 Outward Transport of C6-NBD-PS by BCRP

The transport of C6-NBD-PS and its metabolic products from the inner to the outer plasma membrane leaflet was determined after intracellular labelling of EPG85-257 cells with C6-NBD-PS. Conversion of C6-NBD-PS to other lipid species was not analyzed.
In *BCRP* overexpressing cells, 53% of C6-NBD-lipid was extracted into the medium after a 30 minute incubation at 37°C.

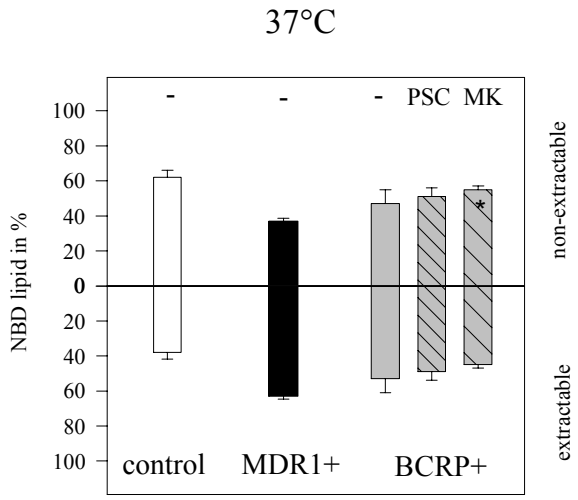


Fig.31: C6-NBD-PS outward transport in controls, *MDR1* overexpressing cells, and *BCRP* overexpressing EPG85-257 cells.

Cells were preincubated for 10 minutes on ice in the absence of MDR inhibitors or in the presence of PSC 833 or MK 571, labelled with C6-NBD-PS and incubated at 20°C for 30 minutes to allow intracellular accumulation of the NBD analog. C6-NBD-PS remaining on the cell surface was then extracted twice by incubation with 2% (w/v) BSA in mPBS for 10 minutes on ice. Then, cells were incubated at 37°C for 30 minutes. Fluorescent lipids (C6-NBD-PS and metabolites) appearing on the cell surface were determined as described in chapter 5. Data represent the means ± S.E.M. of at least n = 2 independent experiments in duplicate, for MK 571 and PSC 833 mean ± range of n = 1 experiments in duplicate. For *BCRP* overexpressing cells, the asterisks denote a significant difference compared to the *BCRP* overexpressing subline without drug pretreatment ($p<0.05$).

This was significantly more than in control cells (38%) (Fig.31).

Treatment with the MDR1 Pgp inhibitor PSC 833 caused a slight decrease in the amount of C6-NBD lipid in the BSA medium of *BCRP* overexpressing cells (49% of C6-NBD lipid extractable by BSA), whereas treatment with the MRP1 inhibitor MK 571 led to a somewhat stronger decrease (45% of C6-NBD lipid extractable by BSA). Fluorescence microscopy confirmed increased outward transport of C6-NBD lipid in *BCRP* overexpressing cells (not shown).

BCRP+

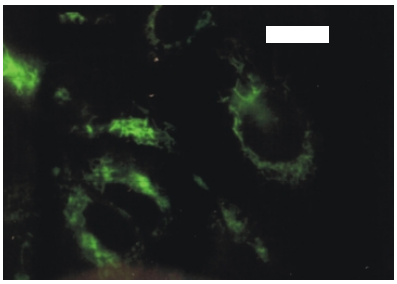


Fig.32: C6-NBD-PS labelling of *BCRP* overexpressing EPG85-257 cells, fluorescence microscopy.

After labelling of the plasma membrane with C6-NBD-PS, the analog was allowed to accumulate intracellularly. For microscopic examination of *BCRP* overexpressing cells, BSA was removed and cells were washed twice. Bar, 20 µm.

Similar to *MDR1* overexpressing cells, C6-NBD lipid fluorescence in the cytosol of *BCRP* overexpressing cells dominated in granular or serpentine structures (Fig.32), presumably representing mitochondria. Unlike control cells (chapter 6.6, Fig.26), neither *MDR1* overexpressing nor *BCRP* overexpressing cells exhibited C6-NBD-lipid fluorescence in a globular region near the nucleus.

7.3 Increased Exposure of Endogenous PS on *BCRP* Overexpressing Cells

Using the PS binding protein conjugate FITC-Annexin V, exposure of endogenous PS was assessed by flow cytometry in *BCRP* overexpressing cells. From the 10 000 single cells counted per sample, damaged cells were excluded using the membrane-impermeable nucleic acid stain propidium iodide. With identical settings, the mean FITC-Annexin V fluorescence intensity (mean ± S.E.M.) was (37 ± 4) a.u. for *BCRP* overexpressing cells, (21 ± 5) a.u. for control cells and (47 ± 5) a.u. for *MDR1* overexpressing cells (Fig.33). Experiments were performed in duplicate. Preincubation of *BCRP* overexpressing cells with the BCRP inhibitor Tryprostatin A reduced the level of FITC-Annexin V binding to (26 ± range 10) a.u..

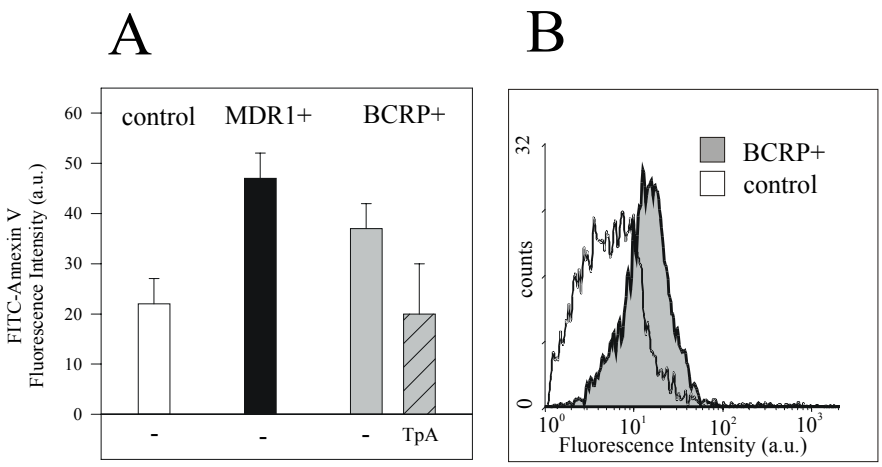


Fig.33: Exposure of endogenous PS on *BCRP* overexpressing and control cells.

Flow cytometric analysis of FITC-Annexin V binding to the cell surface of *MDR1* overexpressing, *BCRP* overexpressing and control human gastric carcinoma EPG85-257 cells. Following a 30 minute preincubation with or without Tryprostatin a (TpA) at 37°C, cells were colabelled with FITC-Annexin V and the membrane impermeable nucleic acid stain propidium iodide as described in chapter 5. Cells showing elevated propidium iodide staining were excluded. 10 000 cells were counted per sample. In the bar plot (A), mean ± S.E.M. of at least n = 2 (TpA: mean ± range of n = 1) independent experiments performed in duplicate are represented, in the histogram (B), FITC-Annexin V binding to *BCRP* overexpressing cells (gray) and control cells (white) is shown in one typical experiment.

7.4 Discussion

Indirect evidence has recently pointed to a potential involvement of the plasma membrane located (Rocchi, 2000) half-size transporter BCRP in the transport of lipid analogs. In the *BCRP* overexpressing human gastric carcinoma subline EPG85-257RN, increased outward transport of C6-NBD-PS was found to coincide with elevated synthesis of BCRP compared to the control. However, synthesis of MDR1 Pgp and MDR1 are also elevated in this subline. Inhibitors of MDR1 Pgp (PSC 833) and MRP1 (MK 571) restrained the outward transport of C6-NBD-PS in *BCRP* overexpressing cells only partially. PSC 833 has been reported not to inhibit BCRP in the literature (Ma, 1998).

Further studies on *BCRP* overexpressing EPG85-257 cells (Woehlecke, 2002) revealed a significant decrease of C6-NBD-PS outward transport in the presence of the BCRP inhibitors Tryprostatin A (Woehlecke, 2002) and GF 120918 (de Bruin, 1999).

Supporting the findings reported in this chapter, this suggests BCRP to be involved in increased outward transport of C6-NBD-PS in *BCRP* overexpressing EPG85-257 cells. In addition, BCRP mediated transport of amphiphilic substrates appears not to be limited to lipid analogs alone. The increased exposure of endogenous PS which was observed in *BCRP* overexpressing cells, and its reduction by the BCRP inhibitor Tryprostatin A, provide evidence for the transport of endogenous PS by BCRP. However, an involvement of MDR1 Pgp and MRP1 and of vesicular transport can not be totally excluded in the lipid transport processes in this cell line.

8 Lipid Transport in the Mammalian Cell Lines LLC-PK1, MDCK II, MF and KPG7

Although an asymmetrical lipid transverse distribution was found in the plasma membranes of various eukaryotic cells, cells originating from different tissues or animal species can show variations in lipid transport and transmembrane distribution.
One of the lipids reported to exhibit a distinctly asymmetrical transverse distribution in the plasma membrane of many cells is the aminophospholipid PS.
In this chapter and chapter 6, cell lines derived from three types of tissue (gastric epithelium (chapter 6), kidney epithelium, fibroblast connective tissue) and four species (human, porcine, canine, murine) are used to gain insight into C6-NBD-PS transport in different mammalian cells.
In subchapter 8.1, C6-NBD-PS outward transport is regarded in the kidney epithelial cell lines LLC-PK1 (porcine) (Hull, 1976) and MDCK II (canine) (Madin, 1958), and in the fibroblast cell lines MF (murine) (Allen, 1999) and KPG7 (human) (Pomorski, 1996) (see chapter 5.3 for cell line origin).
Subchapter 8.2 focuses on outward transport of C6-NBD-PS in the presence of MDR1 Pgp (*MDR1* transfected LLC-PK1 cells) (Schinkel, 1995), respectively in the absence of its mouse homologs Mdr1a/1b Pgp (MF cells with knocked-out *Mdr1a/1b* and *MRP1*) (Allen, 1999). In *MDR1* transfected LLC-PK1 cells, MDR1 Pgp activity is evaluated by assessment of C6-NBD-SM and -GlcCer outward transport (van Helvoort, 1996).

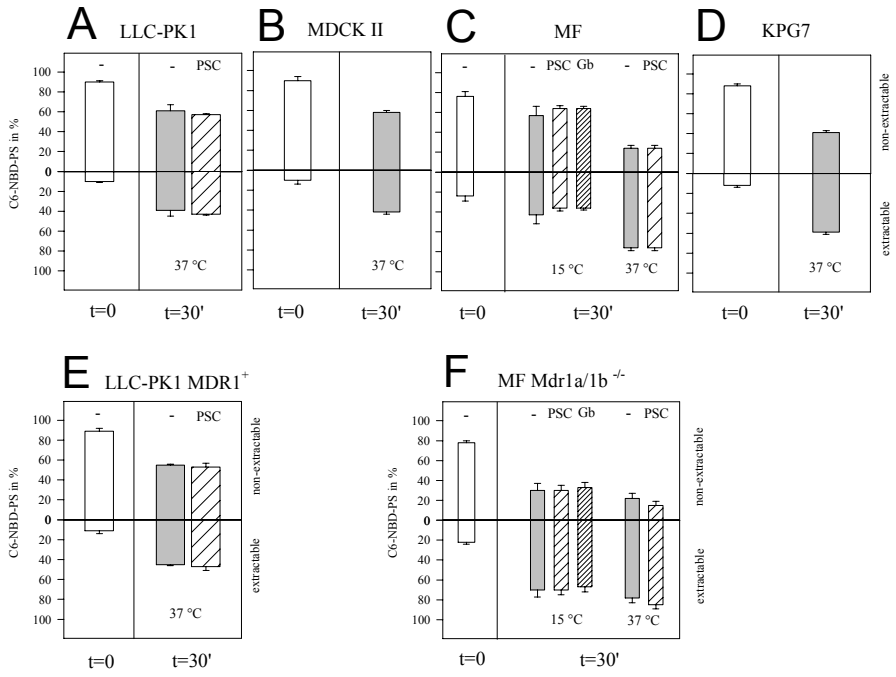


Fig.34: C6-NBD-PS outward transport in four mammalian cell lines.

Pig kidney LLC-PK1 (A, E), canine kidney MDCKII (B), murine fibroblast MF (C, F) and human fibroblast KPG7 (D) cells were preincubated for 10 minutes on ice without inhibitors or with 20 µM PSC 833 or 100 µM Glyburide (Gb), labelled with 5 µM C6-NBD-PS on ice and incubated at 20°C for 30 minutes to allow intracellular accumulation of the NBD analog. Then, C6-NBD-PS remaining on the cell surface was extracted twice by 10 minute incubations with 2% (w/v) BSA in mPBS on ice. Subsequently, cells were incubated at 37°C or 15°C for indicated lengths of time in the presence of 2% BSA to extract C6-NBD-PS and its metabolites appearing on the cell surface. Lipids were analyzed as described in chapter 5. Data represent mean ± range of n = 1 experiment in duplicate for LLC-PK1 and MF, mean ± S.E.M. of n = 2 independent experiments in duplicate for KPG7, mean ± S.E.M. of n = 4 independent experiments in duplicate for MDCK II.

8.1 Outward Transport of C6-NBD-PS

The transport of C6-NBD-PS back to the surface of cells grown on petri dishes was assessed after intracellular labelling with C6-NBD-PS. Cells were subjected to BSA extraction either directly after inward transport (t=0'), or during a 30 minute incubation at 37°C (t=30'). Lipid analogs were separated by 2-D TLC.

Outward transport of C6-NBD-PS in LLC-PK1 pig kidney epithelial cells

In LLC-PK1 cells, 10% of C6-NBD-PS were extracted into the BSA medium immediately after C6-NBD-PS inward transport and BSA extraction from the outer leaflet of the membrane (Fig.34A). After 30 minutes at 37°C, this percentage increased, 39% of C6-NBD-PS had reached the medium.

Outward transport of C6-NBD-PS in MDCK II canine kidney epithelial cells

In MDCK II canine kidney epithelial cells, 10% of C6-NBD-PS were extracted into the BSA medium following inward transport and extraction from the outer leaflet (Fig.34B). A 30 minute incubation at 37°C increased the percentage of extractable C6-NBD-PS to 48%. During C6-NBD-PS outward transport experiments, metabolic conversion of C6-NBD-PS was extensive in MDCK II cells: After the 37°C incubation, 42% of C6-NBD-PS had been converted to other lipid species (-PE: 19%, -FA: 12%, -PA: 11%).

Outward transport of C6-NBD-PS in MF mouse fibroblasts

In MF cells, about 24% of C6-NBD-PS could already be extracted immediately after inward transport and BSA extraction (Fig.34C), possibly due to slight warming of the samples. After a 30 minute incubation at 37°C, 76% of the analog were extractable in MF cells, while only 43% of C6-NBD-PS were found in the medium after a 30 minute incubation at 15°C.

Outward transport of C6-NBD-PS in KPG7 human fibroblasts

In KPG7 cells, 12% of C6-NBD-PS reached the BSA medium when cells were immediately incubated on ice after C6-NBD-PS inward transport and BSA extraction (Fig.34D). During a 30 minute incubation at 37°C, 59% of C6-NBD-PS were extracted into the medium.

8.2 Outward Transport of C6-NBD-Lipids in the Presence and Absence of *MDR1*, Respectively *Mdr1a/1b*

In EPG85-257 cells (chapter 6), *MDR1* overexpression was obtained through in vitro selection with the cytostatic drug daunorubicin (Lage, 2000). Selection often yields cells with a high copy number of MDR1 Pgp, but can result in undesirable additional genetic changes due to long-time selection with a mutagenic substance.
MDR1 expression can also be obtained by transfection.This has the advantage of producing cells which are well-defined on the genetic level, however, *MDR1* expression might be rather low when this technique is used.
In order to obtain a negative control for the MDR1 Pgp homologs Mdr1a/1b Pgp in the mouse, these genes can be knocked out in mice originally expressing both genes.

8.2.1 Outward transport of C6-NBD-lipids in *MDR1* transfected LLC-PK1 cells

MDR1 Pgp was described to mediate outward transport of C6-NBD-GlcCer (van Helvoort, 1996). In order to confirm functionality of MDR1 Pgp in *MDR1* transfected LLC-PK1 cells, apical outward transport of C6-NBD-SM and -GlcCer was studied in polarized LLC-PK1 cells grown on polycarbonate filters with access to both faces. The information on a transport process occurring basolaterally or apically can help to assign a transport activity to a protein. In polarized epithelial cells, MDR1 Pgp was shown to be localized apically (Thiebaut, 1987), MRP1 basolaterally (Evers, 1996).
LLC-PK1 cells grown on petri dishes were used to study the effect of *MDR1* transfection on C6-NBD-PS outward transport. When grown to confluence on petri dishes, LLC-PK1 cells form a polarized monolayer with the apical membrane oriented towards the medium. Therefore, with some caution, the transport measured here can be attributed to the apical domain of the membrane.

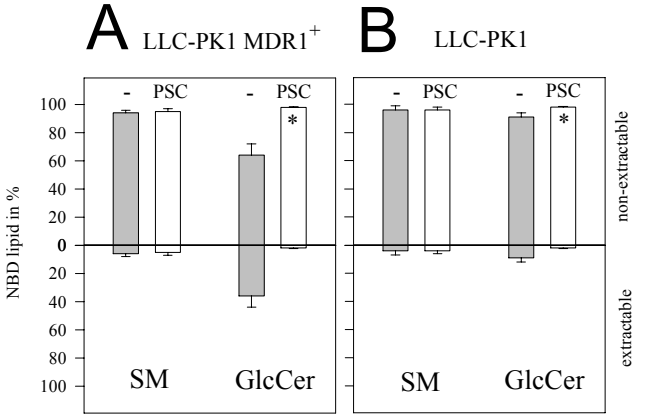


Fig.35: Outward transport of C6-NBD-SM and -GlcCer
in *MDR1* overexpressing and control pig kidney LLC-PK1 cells.

LLC-PK1 cells were preincubated for 10 minutes on ice with or without 20 µM PSC 833, followed by an incubation with 5 µM C6-NBD-Cer for 180 minutes at 15°C in the presence or absence of PSC 833. To extract the fluorescent lipid products (C6-NBD-SM and C6-NBD-GlcCer) appearing on the cell surface, incubation was performed in the presence of 1% BSA. Lipids were quantified as described in chapter 5. Data represent mean ± S.E.M. of n = 2 independent experiments in duplicate. Asterisks denote a significant difference compared to the respective cells without inhibitor.

Outward transport of C6-NBD-GlcCer but not of C6-NBD-SM in MDR1 transfected LLC-PK1 cells

In the course of a 180 minute incubation at 15°C, part of the precursor lipid C6-NBD-Cer added to the cells was converted to C6-NBD-SM and -GlcCer (In chapter 6.5, synthesis and transport processes involved are described in detail). During the incubation, only 6% of C6-NBD-SM, but 36% of C6-NBD-GlcCer were extracted into the apical BSA medium of *MDR1* transfected LLC-PK1 cells (Fig.35) (values refer to the respective lipid analog present in cells and apical medium as 100%). In control samples, very small percentages of each lipid (4% of C6-NBD-SM and 9% of C6-NBD-GlcCer) were found in the apical medium. The already low outward transport of C6-NBD-SM was not influenced by the MDR1 Pgp inhibitor PSC 833 in *MDR1* transfected cells (5% of C6-NBD-SM in the apical medium), or in controls (4% of C6-NBD-SM in the apical medium).

However, PSC 833 strongly reduced outward transport of C6-NBD-GlcCer in *MDR1* transfected cells (2% of C6-NBD-GlcCer in the apical medium), but only slightly in control cells (2% of C6-NBD-GlcCer in the apical medium). Transport of lipids to the basolateral leaflet of the plasma membrane was low in both *MDR1* transfected and control cells, less than 10% of the synthesized C6-NBD-GlcCer and C6-NBD-SM being extracted into the basolateral BSA medium.

Outward transport of C6-NBD-PS in MDR1 transfected *LLC-PK1 cells*
At t=0', 11% of C6-NBD-PS could be extracted into the BSA medium of *MDR1* transfected LLC-PK1 cells (Fig.34E), similar to controls. 45% of C6-NBD-PS reached the medium after a 30 minute incubation at 37°C, which was little more than in controls. PSC 833 had only a slight effect on C6-NBD-PS outward transport in *MDR1* transfected LLC-PK1 cells and controls (47% and 43% of C6-NBD-PS extracted into the medium, respectively).

8.2.2 Outward transport of C6-NBD-PS in *Mdr1a/1b* knock-out MF cells

In *Mdr1a/1b* knock-out MF cells, 22% of C6-NBD-PS were extracted at t=0', near to extraction in controls (Fig.34F). At 37°C, the amount of C6-NBD-PS extracted during a 30 minute incubation was similar for *Mdr1a/1b* knock-out cells (78% extracted) as for controls. In the presence of PSC833, the amount of C6-NBD-PS extractable into the medium of *Mdr1a/1b* knock-out cells appeared to be even slightly increased.
While the amount of extractable C6-NBD-PS strongly decreased in the control line at 15°C, it remained surprisingly high in the *Mdr1a/1b* knock-out MF subline (70% in the medium). Addition of PSC833 did not, and addition of Glyburide (67% of C6-NBD-PS in the medium) did only slightly affect the amount of C6-NBD-PS extracted from *Mdr1a/1b* knock-out cells. In control cells, the percentage of extractable C6-NBD-PS was likewise changed only slightly by the MDR1 Pgp inhibitor PSC833 and the ABCA1 inhibitor Glyburide (36% in the presence of either inhibitor).

8.3 Discussion

8.3.1 Comparison of C6-NBD-PS outward transport in five cell lines

The five mammalian cell lines in which C6-NBD-PS outward transport was assessed in this work vary in their histological origin (gastric epithelium, kidney epithelium, fibroblast connective tissue) as well as in their species affiliation (human, porcine, canine, murine). C6-NBD-PS outward transport varies quite strongly between the different cell lines and appears to be most similar in cell lines with matching histological background.

During a 30 minute incubation at 37°C, outward transport of C6-NBD-PS was almost alike in the two kidney epithelial cell lines studied in this chapter (porcine kidney LLC-PK1 and canine kidney MDCK II, both of mesodermal origin). It was slightly higher in the kidney epithelial lines compared to the gastric epithelial line (human EPG85-257, entodermal origin) used in chapter 6.

In comparison to all epithelial cell lines studied here, C6-NBD-PS outward transport proved to be highly increased in the fibroblast cell lines (murine MF, human KPG7, both of mesodermal origin) at 37°C. At 15°C, where vesicular transport is largely inhibited (van Genderen, 1995), C6-NBD-PS outward transport was reduced in MF fibroblasts, but still surpassed by far transport seen in the human gastric epithelial cell line EPG85-257.

In the cell lines used in this chapter (porcine kidney epithelial LLC-PK1, canine kidney epithelial MDCK II, murine fibroblast MF, human fibroblast KPG7), solely outward transport of C6-NBD-PS was studied, while the effect of an inward transport of this analog was only assessed in human gastric epithelial EPG85-257 cells (chapter 6.6).

For C6-NBD-PS, simultaneous inward transport can be excluded during the outward transport incubation in the presence of BSA, but not for endogenous PS, where lipid inward transport might compensate for modifications in outward transport.

8.3.2 C6-NBD-PS outward transport in the presence or absence of *MDR1*, respectively *Mdr1a/1b*

In the present chapter, increased outward transport of C6-NBD-GlcCer, and its complete inhibition by PSC833, confirms the presence of active, apically localized MDR1 Pgp in *MDR1* transfected LLC-PK1 cells (van Helvoort, 1996). The values obtained here for C6-NBD-GlcCer outward transport correspond very closely with those in the cited work. In agreement with the findings by van Helvoort et al., almost all of the synthesized C6-NBD-SM remained in the cell, as it was trapped in the lumen of the Golgi apparatus in both cell lines.

Outward transport of C6-NBD-SM and -GlcCer is relatively moderate in *MDR1* transfected LLC-PK1 cells in comparison with *MDR1* overexpressing EPG85-257 cells (chapter 6.5), in which the percentage of C6-NBD-SM and -GlcCer reaching the medium within 180 minutes at 15°C is 3.5 fold, respectively 2.3 fold higher. Although the copy number of MDR1 Pgp has not been determined in the two different cell lines, the results obtained with C6-NBD-SM and -GlcCer suggest a higher expression of *MDR1* in *MDR1* overexpressing EPG85-257 cells relative to *MDR1* transfected LLC-PK1 cells (chapter 6.5).

In chapter 6.7, MDR1 Pgp was shown to mediate outward transport of C6-NBD-PS in *MDR1* overexpressing EPG85-257 cells. In the present chapter, outward transport of C6-NBD-PS was found to be slightly increased in *MDR1* transfected LLC-PK1 cells versus controls. However, PSC 833 did not lead to a decrease in the percentage of C6-NBD-PS reaching the medium of these cells. As the same concentrations of PSC 833 fully inhibited MDR1 Pgp mediated transport of C6-NBD-GlcCer, active MDR1 Pgp does not appear to influence C6-NBD-PS outward transport in *MDR1* transfected LLC-PK1 cells.

In MF mouse fibroblasts, outward transport of C6-NBD-PS was extensive at 37°C, yet it was similarly high in *Mdr1a/1b* knock-outs. At 15°C, C6-NBD-PS outward transport was reduced in control cells as would be anticipated. Unexpectedly, however, *Mdr1a/1b* knock-out cells showed even higher outward transport of C6-NBD-PS at this temperature than control cells expressing *Mdr1a/1b*.

While the absence of *Mdr1a/1b* thus appears not to decrease C6-NBD-PS outward transport in *Mdr1a/1b* knock-out cells, it is unclear which transporter(s) are responsible for the high C6-NBD-PS outward transport activity in MF cells. Possibly, the respective proteins are up-regulated compensatorily for the knocked-out ABC proteins in the *Mdr1a/1b, MRP1* knock-out line, working in particular at lower temperatures.

9 Conclusions

The ABC transporter MDR1 Pgp has been implicated with the transport of lipids from the inner to the outer leaflet of the plasma membrane: In 1996, van Helvoort et al. used a porcine epithelial cell line transfected with *MDR1* to demonstrate outward transport of fluorescent short chain (C6-NBD) lipid analogs as models for the behavior of endogenous lipids. Works by Bosch et al. (Bosch, 1997), Ernest and Bello-Reuss (Ernest and Bello-Reuss, 1999), Raggers et al. (Raggers, 2000), Rothnie et al. (Rothnie, 2001) and Romsicki and Sharom (Romsicki, 2001) on MDR1 Pgp mediated lipid transport followed.
A human gastric epithelial cell line (EPG85-257) and its *MDR1* overexpressing subline selected in vitro were employed in the present work. Experiments using C6-NBD-PC, -PE, -SM and -GlcCer confirm recognition of a broad spectrum of lipid analogs by MDR1 Pgp, and demonstrate high activity of MDR1 Pgp in the *MDR1* overexpressing human gastric epithelial cell line.
Surprisingly, the experiments strongly suggest in addition the outward transport of C6-NBD-DG and -Cer by MDR1 Pgp, which have not been reported to be MDR1 Pgp substrates before. Unlike SM and GlcCer, DG and Cer would only be transiently excluded from the cell when transported to the outer leaflet of the plasma membrane, as they can flip back to the inner leaflet within a relatively short time due to their lack of a hydrophilic headgroup.
Transport of endogenous DG, Cer, SM and GlcCer by MDR1 Pgp could be of major consequences for the cell's further destine, as these lipids are implicated in signalling pathways involved in mitosis, cell growth, inflammation and apoptosis (Perry, 1998), (Sietsma, 2001), (Spiegel, 1996), (Fig.36). Briefly, Cer is considered pro-apoptotic, pro-inflammatory and anti-proliferative, DG anti-apoptotic and GlcCer proliferative, while SM is a reservoir for the formation of Cer. However, sphingolipid signalling is only beginning to be elucidated in detail.

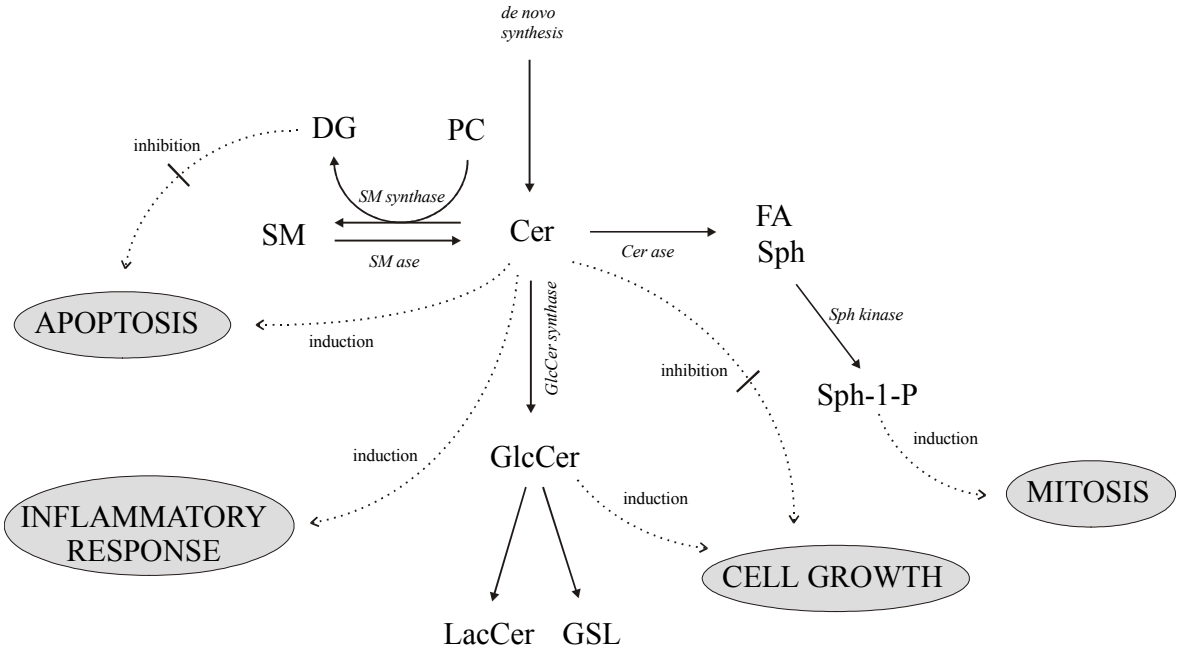


Fig.36: Potential roles of sphingolipids in cell signalling.

Ceramide (Cer) was shown to induce apoptosis in various cells. While the effectors involved are not known in detail, caspases were postulated to be linked to Cer induced apoptosis (Qi, 2001). However, in another work (Watts, 1999), Cer elevation was considered to be a result of and not a cause for apoptosis. The ceramide activated protein (CAP) kinase is believed to lead to an inflammatory response through activation of Raf and subsequently the MAP kinase cascade (Yao, 1995). Potentially through dephosphorylation of the retinoblastoma gene product (Rb) (Dbaibo, 1995), the ceramide activated protein phosphatase (CAPP) is thought to inhibit cell growth (Fishbein, 1993). Diacylglycerol (DG),
on the other hand, inhibits ceramide induced apoptosis (Jarvis, 1994), e.g. on the level of the SMase and presumably further downstream. Glucosylceramide (GlcCer) was reported to stimulate cell growth (Li, 2000). Sphingosine-1-phosphate (Sph-1-P) is a potential mitogen (Spiegel, 1996) and is thought to reverse the apoptotic effects of Cer upstream of caspase activation (Cuvillier, 1996). Sphingomyelin (SM) functions as a reservoir for the various lipid messengers.
The cellular response depends on the particular activities of enzymes regulating the ratio of one sphingolipid species to another and on the equipment of the cell with lipid messenger effectors, but also on the intracellular location of the respective lipid messenger (Perry, 1998). Interestingly, when cellular levels of antagonistic messengers are elevated, as for DG and Cer, there appears to be cross-talk between the two, leading to a differentiated response (Spiegel, 1996).

Besides the relative amounts of the different lipid messengers and the enzymes and effector molecules involved in sphingolipid signalling in the cell, the intracellular localization of the respective lipid second messengers appears to be of importance for the ensuing response (e.g., while both Cer derived from SM on the inner leaflet of the plasma membrane, and Cer generated de novo on the ER, were shown to induce apoptosis, the effector mechanisms are believed to diverge for the two signalling pools, concerning for example the activation of caspases (Perry, 1998)). Outward transport of DG and GlcCer could stimulate apoptosis and defavor cell growth, disadvantageous for further proliferation (Fig.37). Possibly, though, the cell is able to compensate for increased outward transport of GlcCer by alterations in sphingolipid metabolism, e.g. by increased GlcCer formation. Sphingolipid metabolism appears to be rather flexible, illustrated for example by increased conversion of Cer to GlcCer following an experimentally induced elevation in cellular Cer in a colon carcinoma cell line (Veldman, 1998). In fact, the level of GlcCer has been shown to be consistently elevated in several *MDR1* overexpressing cell lines, the level of Cer remaining unchanged (Lavie, 1996), (Sietsma, 2001). In addition, multidrug resistant cells were reported to be highly sensitive to Cer glucosylation inhibitors (Rosenwald, 1994). While this has been previously interpreted as apoptosis due to Cer accumulation, it is also conceivable that due to GlcCer outward transport, the low amount of intracellularly available GlcCer is in part responsible for this phenomenon. Anthracyclines, like several other cytostatic drugs, induce de novo synthesis of Cer (Bose, 1995). Therefore, transport of Cer and SM from the inner to the outer plasma membrane leaflet might be a measure for tumor cells to evade Cer induced apoptosis and to stimulate cell growth. While Sietsma et al. have suggested SM outward transport to prevent the initiation of Cer mediated apoptosis (Sietsma, 2001), direct removal of Cer from the cell interior could constitute a new important mechanism of MDR not previously described. Such a mechanism might explain the strong elevation of the resistance factor against a particular drug, e.g. the anthracycline doxorubicin, in the presence of MDR1 Pgp (Lage, 2000), going along with a comparatively slight reduction in the accumulation of the drug (Stein, 2002). Among the various phospholipids of the plasma membrane, PS occupies a particular place: Under normal conditions, it is confined almost exclusively to the cytoplasmic leaflet.

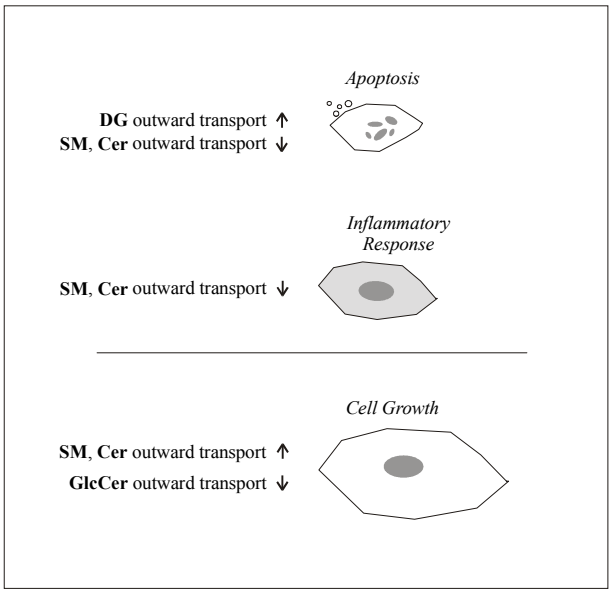


Fig.37: Possible effects of lipid outward transport on cell signalling.

Remarkably, a number of tumor cell lines display 3 to 7-fold elevated amounts of PS on the exoplasmic membrane leaflet compared to non-tumorigenic cells (Utsugi, 1991), which could result from impaired inward transport and/or increased outward transport. As MDR1 Pgp is present in high amounts in the plasma membrane of many tumor cells, this opened up the question whether MDR1 Pgp could be involved in the exposure of endogenous PS. Previously, an analog of PS was claimed not to be an MDR1 Pgp substrate, which has been attributed to the negative charge of this lipid (Bosch, 1997).

In the present work, the combination of the BSA back exchange technique with C6-NBD-PS inward transport by an aminophospholipid translocase activity was established as a new tool to obtain intracellular labelling with the analog, permitting C6-NBD-PS outward transport analyses. In EPG 85-257 human gastric carcinoma cells, MDR1 Pgp was shown to transport C6-NBD-PS from the inner to the outer leaflet of the plasma membrane, while transport of the analog by MRP1, ABCA1 or through vesicles could be excluded. This has not been reported previously. As analogs of endogenous lipids, short-chain NBD lipids only reach a certain degree of accordance due to the short fatty acid chain and the presence of a bulky NBD reporter group.

Nevertheless, since van Helvoort et al. (van Helvoort, 1996) could demonstrate transport of short-chain analogs of PC, PE, and glucosylceramide missing the NBD group in their *MDR1* transfected cell line, transport by MDR1 Pgp does not appear to be due to the modification on the level of one of the fatty acid chains. Recently, Ernest et al. (Ernest and Bello-Reuss, 1999) have shown 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, the platelet activating factor (PAF), to be an endogenous substrate of MDR1 Pgp in human mesanglial cells. Raggers et al. suggested potential outward transport of endogenous GlcCer by MDR1 Pgp (Raggers, 2000).

In the present study, increased amounts of endogenous PS were indeed found to be exposed on the surface of *MDR1* overexpressing cells. This amount could be decreased by an MDR1 Pgp inhibitor, implying for the first time a role for MDR1 Pgp in the recognition and outward transport of endogenous PS.

It remains to be demonstrated whether outward transport of PS in *MDR1* overexpressing cells is sufficient to trigger subsequent biological processes. As outlined in chapter 2.4, cell surface exposure of PS possessing a small, negatively charged headgroup favors cell-cell adherence, recognition and elimination by macrophages, while it can decrease exo- and endocytosis.

In 1996, van Helvoort et al. speculated on the ability of MDR1 Pgp to transport endogenous (aminophospho-) lipids, coming to the conclusion that this is unlikely, as it might lead to a futile ATP utilizing cycle when the same substrates transported inside by the aminophospholipid translocase are moved in the opposite direction by MDR1 Pgp (van Helvoort, 1996). However, *MDR1* has only a low expression in most cells (Cordon-Cardo, 1990), where MDR1 Pgp would not be an important antagonist to the aminophospholipid translocase. In several works, *MDR1* expression was found to become increased in the presence of cytostatic drugs (Hu, 1995). One might speculate that the regulation of MDR1 Pgp according to the current demand could make PS transport an (energetically) expensive side-effect of MDR1 Pgp's detoxifying action, tolerable only when limited in time. In some cell types with moderate *MDR1* expression, MDR1 Pgp mediated outward transport of endogenous PS or other lipids could be a desired function, contributing to the balance of lipid transverse movement across cellular membranes.

It is conceivable that the aminophospholipid translocase activity could be down-regulated in such cells to avoid futile energy consumption (see (Hanson, 2001) for an example of controlled regulation of lipid in- and outward transport in yeast). In fact, without coordinate regulation of the two transporters, high expression of *MDR1* could lead to excessive energy consumption by antagonistic transport of the same substrate. An elevated rate of glucose uptake is typically found in many tumor cells (Kawamura, 2001). Tumor cells, different from untransformed cells, might keep up their resistance against cytostatic drugs by accelerated energy metabolism, as one of a number of instances where tumors disregard economic restraints.

The structure of MDR1 Pgp as determined by electron microscopy and image analysis (Rosenberg, 2001) suggests a rotating helix flippase transport mechanism. One assumption for a flippase/vacuum cleaner transport process is the integration of the substrate into the membrane, from which it can access the transport protein (chapter 2.5).

All substances shown here to be transport substrates of MDR1 Pgp (Rho123, C6-NBD lipid analogs, and possibly endogenous PS) distributed into membranes, as shown by fluorescence microscopy. The MRP1 substrate GS-MF, in contrast, not effectively transported by MDR1 Pgp, was mainly concentrated in the cytosol. The transport mechanism of MDR1 Pgp might differ from that of the glutathione-conjugate export pump MRP1, however, ample data on MRP1 structure and transport are still warranted.

Another assumption for a flippase/vacuum cleaner mechanism is the potentially reduced importance of the interaction between the substrate and the substrate recognition site. Among the substances demonstrated to be transported by MDR1 Pgp in this work were lipid analogs possessing different backbones, and headgroups with positive, negative or no charge. In particular, the addition of the anionic lipid PS to the circle of MDR1 Pgp lipid substrates further supports the idea of MDR1 Pgp acting as a flippase with extremely low substrate specificity. In contrast, the closely related MDR 2/3 Pgp appears to be quite substrate specific, despite being a flippase. The information obtained so far implies a potential connection of MDR1 Pgp mediated multidrug resistance with the emergence of a different lipid pattern in the outer leaflet of the plasma membrane, altering its surface properties, which could have prominent physiological consequences for the cell.

Such changes in the phenotype of drug-treated cells might be a hallmark of multidrug-resistant tumor cells, and could serve for improved diagnostics and treatment strategies of clinical drug resistance.

Although mice lacking the *MDR1* homolog *Mdr1a* are generally healthy in the absence of drugs, increased occurrence of colitis in these animals (Panwala, 1998) is possibly related to a deregulation of lipid transport. In addition to implications for detoxification via MDR1 Pgp, this should be considered when inhibition of MDR1 Pgp is attempted in chemotherapy.

Besides human gastric epithelial cells, four mammalian cell lines were used in the present study to examine outward transport of C6-NBD-PS. In two of these, the effect of *MDR1* expression was regarded.

With the different physiological functions of tissues, cells have to meet particular requirements, concerning among other things their membrane properties (Zachowski, 1993). Cells of the surface epithelium, arising from different embryonal tissues, cover the outer surface of the body or its lumina (e.g. gastric or kidney epithelia). They have a polarized lateral membrane organization, with differing apical and basolateral domains (Simons, 1988), fulfilling both barrier and vectorial transport functions. Cells derived from the connective tissue (mesodermal origin), adopt different physiological roles, among them the formation of fibers and the synthesis of amorphous intercellular substances (e.g. by fibroblasts). In contrast to surface epithelial cells, fibroblasts do not have lateral macrodomains.

The comparison of C6-NBD-PS outward transport in five cell lines originating from four animal species and three types of tissue does not allow generalization, due to the small number of cell lines tested and the limited data size. However, it is noteworthy that C6-NBD-PS outward transport is most similar in similarly specialized cells (porcine LLC-PK1 and canine MDCK II kidney epithelial cells), and lower in all three epithelial cell lines (LLC-PK1, MDCK II, and human EPG85-257) compared to the fibroblast lines (murine MF, human KPG7). It is tempting to speculate on functional reasons for a differently developed outward transport of PS. In epithelia, cell-cell contacts have to be tightly controlled, and adherence due to exposure of a potentially fusogenic lipid as PS (Zachowski, 1993) could compromise their function as a separatory tissue.

In fibroblasts, on the other hand, exposure of fusogenic lipid species might not be functionally disadvantageous, or attachment to other cells might even be desired.
Unlike in EPG85-257 human gastric carcinoma cells, MDR1 Pgp did not appear to affect C6-NBD-PS outward transport in porcine LLC-PK1 porcine kidney epithelial cells and in MF murine fibroblasts. Although in vitro selected *MDR1* overexpressing EPG85-257 cells are genetically not as well-defined as the transfected or knock-out cells, the high expression of *MDR1* in the former is substantiated by Northern and Western blot and by immuno-flow cytometry (Lage, 2000), (Pohl, 2002), (Stein, 2002), (chapter 6.1). In addition, only inhibitors of MDR1 Pgp, but not inhibitors of MRP1 or ABC1, decrease transport of C6-NBD-PS in *MDR1* overexpressing EPG85-257 cells. Therefore, it appears unlikely that outward transport seen in the *MDR1* overexpressing gastric carcinoma cells could be due to another, yet undetected, transport protein expressed in this cell line which is sensitive to MDR1 Pgp inhibitors.
Meanwhile, different reasons are imaginable why *MDR1* transfected LLC-PK1 cells, unlike *MDR1* overexpressing EPG85-257 cells, do not exhibit elevated outward transport of C6-NBD-PS, and why C6-NBD-PS outward transport is not reduced in *Mdr1a/1b* knock-out cells:
As suggested by outward transport of C6-NBD-SM and -GlcCer, the activity of MDR1 Pgp in *MDR1* transfected LLC-PK1 cells is most likely only about half of that found in *MDR1* overexpressing EPG85-257 cells. If this was the case, outward transport of C6-NBD-PS mediated by MDR1 Pgp could be correspondingly low, and get close to the level of detection. The activity of MDR1 Pgp in control MF cells has not been assessed here, but since *MDR1* has generally no strong expression in connective tissue (Cordon-Cardo, 1990), it can be assumed to be rather low in this fibroblast line.
Additionally, outward transport of C6-NBD-PS by MDR1 Pgp might not be distinguishable against the background of an efficient parallel outward transport activity in MF cells. Due to their low background of C6-NBD-PS outward transport, EPG85-257 cells might be particularly appropriate to study outward transport of C6-NBD-PS by lipid transporters.

In addition to studies on lipid transport via MDR1 Pgp, EPG85-257 human gastric carcinoma cells were used here to study lipid transport via BCRP, another member of the ABC superfamily.

BCRP has only recently been identified, and little has been known thus far on whether it transports lipid substrates. In 2000, a work by Litman et al. (Litman, 2000) implied that BCRP is possibly involved in the transport of a ceramide analog, and H. Woehlecke has recently found active BCRP to increase outward transport of C6-NBD-PC (Woehlecke, 2002). In the present work, BCRP is suggested to mediate outward transport of C6-NBD-PS. Consistently, this transport is reduced by BCRP inhibitors, as demonstrated recently by H. Woehlecke (Woehlecke, 2002). On the outer leaflet of the plasma membrane, *BCRP* overexpressing EPG85-257 cells expose increased amounts of endogenous PS, which appears to be suppressed by an inhibitor of BCRP.

Apparently, thus, the transport of lipids or their analogs is a feature not unique for some ABC full-size transporters only. Instead, it is also found for the ABC half-size transporter BCRP. So far, its mechanism of transport is still open, and it is not unraveled whether transport-active BCRP is made up of homodimers (Ozvegy, 2001) or rather heterodimers. While BCRP appears to transport different lipid analogs, it is not known whether this transporter possesses a similarly broad spectrum of lipid substrates as MDR1 Pgp (as suggested by transport of such different lipids as Cer and PS) and functions as a flippase or vacuum cleaner, or whether it is more selective. Broad substrate specificity could be a feature generally useful for MDR proteins, recognizing a spectrum of unknown substances which, due to their amphiphilic character, are able to reach the cell interior across the hydrophobic core of the membrane, cross the hydrophilic cytoplasm, and eventually reach the nucleus via the nuclear membrane to potentially cause damage, e.g. on the DNA level. Similarly, increased exposure of endogenous PS in the outer plama membrane leaflet of multidrug resistant cells could result from the activity of various MDR proteins, rather than of MDR1 Pgp mediated transport alone.

As for MDR1 Pgp, it will be a challenging task to understand in which way BCRP mediated lipid transport could possibly be used in diagnosis or therapy, and how it might affect the phenotype of atypical multidrug resistance in cancer.

10 Outlook

Various data in the present work suggest MDR1 Pgp to be a flippase with extremely low substrate specificity. In chapter 9, a potential connection between GlcCer outward transport and an increased sensitivity of multidrug resistant cells towards Cer glucosylation inhibitors was proposed. Therefore, it could be of high medical interest to verify endogenous GlcCer as an MDR1 Pgp transport substrate (Raggers, 2000). Due to the importance of DG and Cer in signalling, and potentially in the development of multidrug resistance, additional experiments should be envisaged to directly assess outward transport of these lipids or their analogs via MDR1 Pgp. In cells labelled with C6-NBD-PA or -Cer, a BSA back exchange preceding the outward transport incubation could provide data on the outward transport rather than on the accumulation of C6-NBD-DG and -Cer. In order to determine the cell surface exposure of endogenous DG and Cer, one could e.g. imagine the development protocols based on enzymatic approaches (Jones, 1982) or on labelling by specific antibodies (Vielhaber, 2001). However, in these analyses, the fast redistribution of both DG and Cer across membranes must be taken into account. On the other hand, the fast passive flip-flop of Cer and DG could theoretically make them ideal inhibitors of MDR1 Pgp (Eytan, 1999), keeping MDR1 Pgp busy in a futile transport cycle. However, an artificial increase in Cer levels might induce apoptosis in normal cells, rather than in *MDR1* overexpressing cells where it could be removed from its place of action. DG, on the other hand, is an apoptosis inhibitor, which makes it unsuitable for the reversal of multidrug resistance. As specified in chapter 9, the transverse distribution of endogenous PS over the leaflets of the plasma membrane could notably influence surface properties, cell-cell interactions and exo- and endocytotic processes.

To estimate the part which MDR1 Pgp assumes in the outward transport of endogenous PS, detailed information is needed on the transbilayer dynamics of endogenous PS, e.g. on the transport of this lipid by the aminophospholipid translocase. PS exposed on the surface of *MDR1* overexpressing cells might be recognized by macrophages as a signal for phagocytosis, or be artificially identified and assailed. Despite its slow passive flip-flop compared to DG and Cer, PS might compete for transport with other MDR1 Pgp substrates due to inward transport by the aminophospholipid translocase activity. However, as PS is an endogenous membrane component, this does not appear to lead to major inhibition in most cells expressing *MDR1*. Albeit the broad spectrum of its lipid substrates, different affinities of MDR1 Pgp for various lipid species can give rise to a certain degree of specificity for transport. Detailed studies on the affinity of MDR1 Pgp for lipids are necessary to understand modulation of MDR1 Pgp mediated multidrug resistance by the ubiquitous lipids. Very likely, these questions can be addressed successfully only in a defined, yet adequately physiological system, e.g. transfected cells with a high level of *MDR1* expression and a natural membrane environment, or MDR1 Pgp reconstituted into giant liposomes with a distinct lipid composition and a marginal degree of membrane curvature similar to that of a cell, avoiding the generation of high lateral pressure that could potentially inhibit lipid transport.

For the half-size transporter BCRP, transport of lipids and lipid analogs is starting to be elucidated at present. Still, additional experimental data is needed to support outward transport of endogenous PS via BCRP, and to determine which other lipids might be substrates of this emerging lipid transport protein.

Beyond studying the outward transport of C6-NBD-PS in five different cell lines, comparison of the transverse distribution of various endogenous lipids in these cells and others derived from different species and types of tissue might answer whether the lipid transverse distribution generally depends to a high degree on the type of tissue, or whether PS is an exception in this respect. In the former case, it would be challenging to learn about which roles the transverse asymmetry could assume in tissue specific function.

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Abbreviations

ABC	ATP binding cassette
ANOVA	analysis of variance
BCRP	breast cancer resistance protein
BFA	brefeldin A
Cer	ceramide
CMFDA	5-chloromethylfluoresceine diacetate
ConA	concanavalin A
CsA	cyclosporin A
Dex	dexniguldipine-HCl, B8509-035
DFP	diisopropyl fluorophosphate
DG	diacyl glycerol
EPG85-257	Eppendorf gastric carcinoma 1985 #257
ER	endoplasmic reticulum
FA	fatty acid
FCS	fetal calf serum
GlcCer	glucosylceramide
GS-MF	glutathione-methylfluoresceine
mAb	monoclonal antibody
MDR	multidrug resistance
MDR1 Pgp	MDR1 P-glycoprotein
MDR2/3 Pgp	MDR2/3 P-glycoprotein

MK 571	MRP1 specific inhibitor
mPBS	modified phosphate buffered saline
MRP1	multidrug resistance protein 1
MXR	mitoxantron resistance associated transporter (BCRP)
NBD	[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]
PA	phosphatidic acid
PAF	platelet activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine)
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
PSC 833	MDR1 Pgp inhibitor
Rho 123	rhodamine 123
RT-PCR	reverse transcription polymerase chain reaction
S.E.M.	standard error of mean
SM	sphingomyelin
TGN	trans golgi network
TLC	thin layer chromatography
TM	transmembrane
WGA	wheat germ agglutinin

Lebenslauf

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Publikationen

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Herrmann, A., Libera, J., **Pohl, A.**, Tannert, A., Müller, P. (2001)
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Woehlecke, H., **Pohl, A.**, Lage H., Herrmann, A.
BCRP promotes transport of phospholipids in a human gastric carcinoma cell line.
(in preparation)

Vorträge

16.03.99	Workshop Dynamics and Evolution of Cellular and Macromolecular Processes (Hiddensee) "Transport of Phosphatidylserine (PS) by the Human MDR1 P-Glycoprotein"
23. 02.00	Workshop Theoretical Biophysics (Maribor, Slowenien) "Is there a link between multidrug resistance and disturbed asymmetry?"
22.08.01	International Workshop of Biophysics and Bioinformatics (Berlin) "ABC-Proteins as lipid transporters"

Erklärung

Die vorliegende Promotion habe ich selbständig und ohne unerlaubte Hilfe angefertigt.

Ich besitze keinen entsprechenden Doktorgrad und habe mich anderwärts nicht um einen Doktorgrad beworben.

Die dem Promotionsverfahren zugrundeliegende Promotionsordnung ist mir bekannt.

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